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In re application of:

Mike A. Clark

Serial No.: **10/674,666** Group Art Unit: **1648**

Filing Date: **September 29, 2003** Examiner: **Emily M. Le**

For: **Methods For Inhibiting Viral Replication In Vivo**

DECLARATION OF JOHN S. BOMALASKI UNDER 37 C.F.R. § 1.132

I, John S. Bomalaski, hereby declare that:

1) I am the president and chief medical officer of Phoenix Pharmacologics, Inc. I received a medical degree from St. Louis University in 1978 and was a medical intern and resident at the Northwestern University Medical School from 1978 to 1981. I was the chief medical resident at the Northwestern Memorial Hospital from 1981 to 1982 and was a rheumatology fellow at the University of Pennsylvania from 1982 to 1984. I was an assistant professor of medicine, associate professor of medicine, and professor of medicine at the Medical College of Pennsylvania from 1984 to 1988, 1988 to 1994, and 1995 to 2004, respectively. In 1996 I cofounded Phoenix Pharmacologics, Inc., and I served as the executive vice president and chief medical officer of Phoenix Pharmacologics, Inc. from 1996 to 2004. I have been in my present position at Phoenix Pharmacologics, Inc. since 2004.

2) I have reviewed the above-referenced patent application (copy attached as Exhibit A), and I have also reviewed a set of patent claims (*i.e.*, claims 1 to 19, 22, 25, 41, 42, and 52 to

73, copy attached as Exhibit B) that I understand to be pending in the application. I have further reviewed an Office Action that was issued April 22, 2005 in connection with the application.

3) The claimed subject matter provides, *inter alia*, methods of inhibiting Hepatitis C virus (HCV) replication in an individual that comprise administering to the individual a composition comprising an arginine deiminase (ADI) bonded to polyethylene glycol (PEG) in an amount effective to inhibit HCV replication in the individual.

4) Experiments performed at Phoenix Pharmacologics demonstrate that arginine deiminase bonded to polyethylene glycol (ADI-PEG) inhibits replication of HCV *in vitro* through a mechanism that does not involve the killing of hepatocellular carcinoma (HCC) cells. That is, experiments have been performed at Phoenix Pharmacologics that demonstrate that ADI-PEG inhibits replication of HCV in viable HCC cells *in vitro*.

5) In a particular set of experiments, which are described in Example 8 of the specification, cultures of AVA5 cells[1] were treated with various concentrations of ADI-PEG. The concentration of ADI-PEG required to inhibit 50 % of the HCV viral replication, as measured by HCV mRNA levels (IC_{50}), was determined, as was the concentration of ADI-PEG required to kill 50 % of the AVA5 cells (CC_{50}). The selectivity index (CC_{50}/IC_{50}) was then determined, which is an indicator of the level of inhibition of viral replication that occurs in the absence of the killing of host cells. A selectivity index of greater than 10 indicates that viral replication is selectively inhibited in the absence of host cell killing. As can be see from

[1] A stable HCV RNA cell line derived by transfection of the human heptoblastoma cell line Huh7.

the results presented in Example 8, ADI-PEG inhibits HCV replication with a selectivity index of 12, indicating that the enzyme inhibits HCV replication through a mechanism that does not involve the killing of host cells.

6) Although ADI has been shown to kill HCC cells *in vitro* and *in vivo*[2], it does not necessarily follow, for a number of reasons, that ADI inhibits HCV replication by killing HCC cells. First, HCV replication occurs diffusely throughout the liver, and does not occur only in HCC cells. Most patients that have HCV do not have HCC, and HCC cells are thus not required for HCV replication. Second, ADI has been shown to kill numerous types of tumor cells that do not act as HCV host cells. See, for example, Dillon, B.J., *et al., Cancer*, 2004, 100, 826-833 (copy attached as Exhibit C). Finally, as discussed above, ADI-PEG has been shown to selectively inhibit HCV replication by a mechanism that is independent of the killing of host cells.

7) The results of clinical trials conducted on behalf of Phoenix Pharmacologics, which are presented in Table III of the specification, indicate that a correlation does not exist between the response of HCC tumors to ADI-PEG and the response of HCV to ADI-PEG, providing further evidence that ADI-PEG does not inhibit HCV replication by killing HCC cells. For example, in patient 1, HCC tumors were only modestly reduced by ADI-PEG, but ADI-PEG decreased the HCV titer by over 99 %. In patient 2, the HCC tumors were stable, indicating that they were not affected by ADI-PEG, but the HCV titers decreased 96 % after ADI-PEG treatment. Patient 6 made a complete recovery from HCC with ADI-PEG treatment, but the patient's HCV titers only decreased 47 % following treatment. In patient

[2] Reported, for example, at page 1816 of Izzo, F., *et al., J. Clin. Oncol.*, 2004, 22, 1815-1822.

12, ADI-PEG modestly reduced HCC tumors, while the patient's HCV titers actually increased. The lack of correlation between the response of HCC tumors and HCV titers to ADI-PEG provides further evidence that HCV replication is selectively inhibited by ADI-PEG through a mechanism that does not involve the killing of HCC cells.

8) The results of more recent clinical trials conducted on behalf of Phoenix Pharmacologics indicate that ADI-PEG significantly reduces HCV titers in patients infected with HCV of differing serotypes. Patients suffering from HCC and infected with HCV of various serotypes were administered ADI-PEG. As shown in the table below, in four of twelve patients the HCV titers decreased over 99 % following ADI-PEG treatment. Two of the four patients (patients 1712 and 1906) were infected with HCV serotype 1b and the remaining two patients (patients 1502 and 1802) were infected with HCV serotype 2a/2c.

			HCV Titer		
Patient #	**Anti-HCV Ab**	**HCV genotype**	**Pre ADI-PEG**	**With ADI-PEG**	**Titer % Decrease**
1307	positive		<1.0E3	<1.0E3	0
1402	positive	1b	8.19E+05	3.18E+05	6.10E+01
1403	positive	1b	5.05E+05	2.93E+06	increase
1502	positive	2a/2c	5.15E+05	2.77E+03	>99
1505	positive	1b	1.18E+07	2.56E+06	1.50E+01
1508	positive	2	3.93E+05	6.09E+05	increase
1606	positive	2a/2c;4	2.04E+04	3.53E+04	increase
1704	positive	1	3.95E+05	5.43E+06	increase
1707	positive	6a	2.56E+04	1.85E+05	increase
1712	positive	1b	7.56E+06	ND	>99
1802	positive	2a/2c	1.62E+05	ND	>99
1806	positive	1b	1.19E+07	<1.0E3	>99

9) I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

19 August 2005 John Bomalaski

Date John Bomalaski

METHODS FOR INHIBITING VIRAL REPLICATION *IN VIVO*

[0001] CROSS REFERENCE TO RELATED APPLICATIONS

[0002] The present application claims priority of Application Serial No. 60/427,497, filed November 18, 2002, which is hereby incorporated by reference in its entirety.

[0003] FIELD OF THE INVENTION

[0004] The present invention is directed to methods for inhibiting viral replication, to methods for treating cancer, to methods for treating and/or inhibiting metastasis, and to methods for concurrently inhibiting viral replication and treating cancer or treating and/or inhibiting metastasis, and others.

[0005] BACKGROUND OF THE INVENTION

[0006] Viral infections are among the leading causes of death with millions of deaths each year being directly attributable to several viruses including hepatitis and HIV.

[0007] Hepatitis is a disease of the human liver. It is manifested with inflammation of the liver and is usually caused by viral infections. Several viruses such as hepatitis A, B, C, D, E and G are known to cause viral hepatitis. Among them, HBV and HCV are the most serious.

[0008] Hepatitis C virus (HCV) is pandemic with more than 170 million persons worldwide infected. Among viral diseases, it is 5 times more widespread that human immunodeficiency virus type 1 (HIV-1), and approximately 10,000 Americans will die this year from cirrhosis and hepatocellular carcinoma (HCC) resultant from chronic HCV infection (Sun CA, Wu DM, Lin CC, LU SN, You SL, Wang LY, Wu MH, Chen CJ. 2003. Incidence and cofactors of hepatitis C virus-related hepatocellular carcinoma: a prospective study of 12,008 men in Taiwan. Am J Epidemiol 157:674-682; Herrine SK. 2002. Approach to the patient with chronic hepatitis C virus infection. Ann Intern Med 136: 747-757; Hoofnagle JH. 2002. Course and outcome of hepatitis C. Hepatology 36:S21-S29; Lauer GM, Walker BD. 2001 Hepatitis C virus infection. N Engl J Med 345:41-52; Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. 2001. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 132:296-305). Furthermore, the prevalence of HCV continues to increase in the USA, Western

Europe and Asia despite the institution of blood donor screening programs. Progression to chronic disease occurs in most HCV infected patients. In addition, HCV causes HCC in 1 - 4% annually of all chronically infected individuals. Moreover, HCC can occur even in those without cirrhosis (Shiratori Y, Shiina S, Teratani T, Imamura M, Obi S, Sato S, Koike Y, Yoshida H, Omata M. 2003. Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. Ann Int Med 138:299-306; Smith MW, Yue ZN, Geiss GK, Sadovnikova NY, Carter VS, Boix L, Lazaro CA, Rosenberg GB, Bumgarner RE, Fausto N, Bruix J, Katze MG. 2003. Identification of novel tumor markers in hepatitis C virus-associated hepatocellular carcinoma. Cancer Res 63:859-864; Yoshizawa H. 2002. hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 62 (Suppl 1):8-17; Colombo M. 1999. Natural history and pathogenesis of hepatitis C virus related hepatocellular carcinoma. J Hepatology 31 (Suppl 1):25-30). Given the current prevalence of HCV infection among persons 30 to 50 years of age, the incidence and mortality rates of HCC are estimated to double in the United States over the next 10 to 20 years (El-Serag HB. 2002. Hepatocellular carcinoma and hepatitis C in the United States. Hepatology 36:S74-S83). It is estimated that there are 500 million people infected with it worldwide. No effective immunization is currently available, and hepatitis C can only be controlled by other preventive measures such as improvement in hygiene and sanitary conditions and interrupting the route of transmission.

[0009] Today, there is no effective therapy for HCC except surgical resection (Ryder SD. 2003. Guidelines for the diagnosis and treatment of hepatocellular carcinoma (HCC) in adults. Gut 52 (Suppl III):iii1-iii8; El-Serag HB. 2002. Hepatocellular carcinoma and hepatitis C in the United States. Hepatology 36:S74-S83; El-Serag HB. 2001. Global epidemiology of hepatocellular carcinoma. Clin Liver Dis 5:87-107; DiMaio M, DeMaio E, Perrone F, Pegnata S, Daniele B. 2002. Hepatocellular carcinoma: systemic treatments. J Clin Gastroenterol 35 (Suppl. 2):S109-S114; Curley SA, Izzo F, Ellis LM, Vauthey JN, Vallone P. 2000. Radiofrequency ablation of hepatocellular cancer in 110 patients with cirrhosis. Ann Surg 232:381-391; Watkins KT, Curley Sa. 2000. Liver and bile ducts. In Clinical Oncology, 2nd ed. Editors MD Abeloff, JO Armitage, AS Lichter, JE Niederhuber. New York: Churchill Livingstone, pp.1681-1748). However, only < 5 % of HCC patients are surgical candidates and only ~1% actually undergo resection. Even among those resected, recurrence of HCC is common, especially in those infected with HCV.

[0010] Amino acid deprivation therapy is an effective means for the treatment of some cancers. Although normal cells do not require arginine, many cancer cell lines are auxotrophic for this amino acid. Thus, cancers, including but not limited to HCC, may be selectively killed by arginine deprivation therapy (Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA. 2002. Pegylated arginine deiminase (ADI-SS PEG $_{20,000}$ mw) inhibits human melanomas and hepatocellular carcinomas *in vitro* and *in vivo*. Cancer Res 62:5443-5440; Takaku, H, Misawa, S, Hayashi H and Miyazaki K. (1993). Chemical modification by polyethylene glycol of the anti-tumor enzyme arginine deiminase from *Mycoplasma arginini*. Jpn. J. Cancer Res. 84:1195-1200; Takaku H, Takase M, Abe S, Hayashi H and Miyazaki K. (1992). *In vivo* anti-tumor activity of arginine deiminase purified from *Mycoplasma arginini*. Int. J. Cancer 51:244-249; Sugimura K, Ohno T, Kussyama T, Azuma I. 1992. High sensitivity of human melanoma cell lines to the growth inhibitory activity of *Mycoplasma arginini* deiminase *in vitro*. Melanoma Res. 2:191-196). High sensitivity of human melanoma cell lines to the growth inhibitory activity of *Mycoplasma arginini* deiminase *in vitro*. Melanoma Res. 2:191-196). This therapy is well tolerated as arginine is not an essential amino acid in humans (Rose WC. 1949. Amino acid requirements of man. Fed Proc 8:546-452, Snyderman, S., E., Boyer, A., and L.E. Holt 1959. The arginine requirement of the infant. J. Dis. Child. 97:192 and for review see Rodgers QR. 1994. Species variation in arginine requirements. In Proceedings from a Symposium Honoring Willard J. Visek - from Ammonia to Cancer and Gene Expression. Special Publication 86 - April 1994, Agriculture Experiment Station, University of Illinois, 211 Mumford Hall, Urbana, IL 61801, pp. 9-21, as it can be synthesized from citrulline. ADI converts extracellular arginine into citrulline which may be taken up by normal cells and converted into arginine intracellularly but not by cancer cells, especially HCC cells, because they lack the rate limiting enzyme argininosuccinate synthetase (Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA. 2002. Pegylated arginine deiminase (ADI-SS PEG $_{20,000}$ mw) inhibits human melanomas and hepatocellular carcinomas *in vitro* and *in vivo*. Cancer Res 62:5443-5440). This inability to express argininosuccinate synthetase has recently been confirmed by others (Shen LJ, Lin WC, Beloussow K, Shen WC. 2003. Resistance to the anti-proliferative activity of recombinant arginine deiminase in cell culture correlates with the endogenous enzyme, argininosuccinate synthetase. Cancer Lett 191:165-170) We have extended this study of argininosuccinate synthetase deficiency to other tumors (Dillon BJ, Prieto VG, Curley SA, Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA. 2003. The method incidence and distribution of

argininosuccinate synthetase deficiency in human cancers: a method for identifying cancers sensitive to arginine deprivation. Cancer (in press). Preliminary results from human clinical testing of ADI-SS PEG 20,000 mw indicates this therapy to be both safe and effective as an anti-cancer treatment.

[0011] Hepatitis B virus infection can lead to a wide spectrum of liver injury. Moreover, chronic hepatitis B infection has been linked to the subsequent development of hepatocellular carcinoma, a major cause of death. Current prevention of HBV infection is a hepatitis B vaccination which is safe and effective. However, vaccination is not effective in treating those already infected (i.e., carriers and patients).

[0012] Acquired immune deficiency syndrome (AIDS) is a fatal disease, reported cases of which have increased dramatically within the past several years. The AIDS virus was first identified in 1983. It has been known by several names and acronyms. It is the third known T-lymphotropic virus (HTLV-III), and it has the capacity to replicate within cells of the immune system, causing profound cell destruction. The AIDS virus is a retrovirus, a virus that uses reverse transcriptase during replication. This particular retrovirus is also known as lymphadenopathy-associated virus (LAV), AIDS-related virus (ARV) and, most recently, as human immunodeficiency virus (LIV). Two distinct families of HIV have been described to date, namely HIV-1 and HIV-2. The acronym "HIV" is used herein to refer to human immunodeficiency viruses generically.

[0013] Herpes simplex virus (HSV) types 1 and 2 are persistent viruses that commonly infect humans; they cause a variety of troubling human diseases. HSV type 1 causes oral "fever blisters" (recurrent herpes labialis), and HSV type 2 causes genital herpes, which has become a major venereal disease in many parts of the world. No fully satisfactory treatment for genital herpes currently exists. In addition, although it is uncommon, HSV can also cause encephalitis, a life-threatening infection of the brain. (The Merck Manual, Holvey, Ed., 1972; Whitley, Herpes Simplex Viruses, In: Virology, 2nd Ed., Raven Press (1990)). A most serious HSV-caused disorder is dendritic keratitis, an eye infection that produces a branched lesion of the cornea, which can in turn lead to permanent scarring and loss of vision. Ocular infections with HSV are a major cause of blindness. HSV is also a virus which is difficult, if not impossible to cure.

[0014] **Anti-Viral Therapies**

[0015] There are several problems with current anti-viral therapies. First, there are relatively few effective antiviral drugs. Many of the existing anti-virals cause adverse or

undesirable side-effects. Most effective therapies (such as vaccination) are highly specific for only a single strain of virus. Frequently the virus undergoes mutation such that it becomes resistant to either the drug or vaccine.

[0016] Many of the current treatments for viral infections revolve around interferon-α (IFN-α.). It is believed that IFN-α binds to cellular receptors and initiates an intracellular response that includes enzymes involved in protein synthesis. This ultimately leads to the anti-viral activity/response. However, data from various clinical trials have shown that approximately 40% of patients treated with IFN-α initially responded to the therapy, but 70% of these relapsed after the treatment ended. (Damen, M., and Bresters, D., in H. W. (ed.): Curr. Stud. Hematol. Blood Transf., Darger Publishers 1998, Basel.) Overall, the long-term therapeutic effect and response was observed in only 10 to 30% of the patients. (Houghton, M., in Fields, B. N. et al., Fields Virology, Raven Publishers 1996, Philadelphia). In addition many side effects were observed such as severe flu, fatigue, muscle and head aches, even depression, weight loss and diarrhea. (Damen, M., and Bresters, D., in H. W. (ed.): Curr. Stud. Hematol. Blood Transf., Darger Publishers 1998, Basel.)

[0017] HCV therapy

[0018] The current standard therapy for HCV infection is pegylated (PEG) interferon-α (IFN) and ribavirin. Although this therapy can result in sustained anti-viral response, significant numbers of patients do not respond to this therapy or are excluded from this treatment (Falck-Ytter Y, Kale H, Mullen KD, Sarbah SA, Sorescu L, McCullough AJ. 2002. Surprisingly small effect of antiviral treatment in patients with hepatitis C. Ann Intern Med 136:288-292; Fried MW. 2002. Side effects of therapy of hepatitis C and their management. Hepatology 36:S237-S244; Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL Jr, Häussinger K, Diago M, Carosi G, Dhumeaux K, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975-982.; Herrine SK. 2002. Approach to the patient with chronic hepatitis C virus infection. Ann Intern Med 136:747-757; Lauer GM, Walker BD. 2001. Hepatitis C virus infection. N Engl J Med 345:41-52; Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. 2001. Pathogenesis, natural history, treatment and prevention of hepatitis C. Ann Intern Med 132:296-305; Manns MP, McHutchinson JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M-H, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet

358:958-965). For example, recent studies of PEG-IFN α-2a (Pegasys^{TN}) plus ribavirin, and PEG-IFN α-2b (Pegintron^{TN}) plus ribavirin demonstrate that ~ 56% of studied patients had a sustained viral response (Dantzler TD, Lawitz EJ. 2003. Treatment of chronic hepatitis C in nonresponders to previous therapy. Curr Gastroenterol Rep 5:78-85; Masci P, Bukowski RM, Patten PA, Osborn BL, Borden EC. 2003. New and modified interferon alfas: preclinical and clinical data. Curr Oncol Rep 5:108-113; Chandler G, Sulkowski MS, Jenckes MW, Torbenson MS, Herlong HF, Bass EB, Gebo KA. 2002. Treatment of chronic hepatitis C: a systematic review. Hepatology 36:S135-S144; DiBisceglie AM, Hoofnagle JH. 2002. Optimal therapy of hepatitis C. Hepatology 36:S121-127; Fried MW. 2002. Side effects of therapy of hepatitis C and their management. Hepatology 36:S237-S244; Lindsay KL. 2002. Introduction to therapy of hepatitis C. Hepatology 36:S114-S120. López-Guerrero JA, Carrasco L. 1998. Effect of nitric oxide on poliovirus infection of two human cell lines. J Virol 72:2538-2540; Wedemeyer H, Wiegand J, Cornberg M, Manns MP.; Polyethylene glycol-interferon: Current status in hepatitis C virus therapy, J Gastroenterol Hepatol. 2002 Dec; 17 Suppl 3:S344-S350; Manns MP, McHutchinson JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M-H, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet 358:958-965). However, for HCV genotypes 1a and 1b, the most common genotypes in the USA and western Europe, the response was only ~ 46%. HCV genotypes 2 and 3 had a better response (76% - 82%). Furthermore, this response rate of ~50% is only for patients studied in clinical trials; it does not represent the entire patient population and is, therefore, biased ((Dantzler TD, Lawitz EJ. 2003. Treatment of chronic hepatitis C in nonresponders to previous therapy. Curr Gastroenterol Rep 5:78-85; Masci P, Bukowski RM, Patten PA, Osborn BL, Borden EC. 2003. New and modified interferon alfas: preclinical and clinical data. Curr Oncol Rep 5:108-113; Chandler G, Sulkowski MS, Jenckes MW, Torbenson MS, Herlong HF, Bass EB, Gebo KA. 2002. Treatment of chronic hepatitis C: a systematic review. Hepatology 36:S135-S144; DiBisceglie AM, Hoofnagle JH. 2002. Optimal therapy of hepatitis C. Hepatology 36:S121-127; Fried MW. 2002. Side effects of therapy of hepatitis C and their management. Hepatology 36:S237-S244; Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL Jr, Häussinger K, Diago M, Carosi G, Dhumeaux K, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975-982; Lindsay KL. 2002. Introduction to therapy of hepatitis

C. Hepatology 36:S114-S120. López-Guerrero JA, Carrasco L. 1998. Effect of nitric oxide on poliovirus infection of two human cell lines. J Virol 72:2538-2540; Wedemeyer 2002, Manns MP, McHutchinson JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M-H, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet 358:958-965). For example, a large study in the USA excluded 404 out of 1337 (or ~30%) of potential patients due to selection criteria (McHutchinson JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, et al. 1998. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med 339:1485-1492). Other large studies often fail to describe their screening criteria or the percentage of patients enrolled. A recent study performed in the USA by a large teaching hospital noted that 72% of all HCV patients were not treated with IFN for reasons such as medical or psychiatric contraindications, ongoing substance or alcohol abuse, failure to adhere to evaluation procedures, normal liver enzymes or even patient preference of no treatment (Falck-Ytter Y, Kale H, Mullen KD, Sarbah SA, Sorescu L, McCullough AJ. 2002. Surprisingly small effect of antiviral treatment in patients with hepatitis C. Ann Intern Med 136:288-292). Similar results have been confirmed by others (Diamond C, Lee JH. 2002. Use of antiviral therapy in patients with hepatitis C. Annals Intern Med 137:1012). Thus a significant portion of the HCV infected population does not receive current "best standard of care" treatment due to a variety of medical or psychiatric contraindications. Even in studies using the "best" patients in the USA and western Europe, only ~50% achieve sustained viral response.

[0019] IFN-α also has significant side effects which occur with approximately the same frequency in both the PEG and non PEG formulated versions (Masci P, Bukowski RM, Patten PA, Osborn BL, Borden EC. 2003. New and modified interferon alfas: preclinical and clinical data. Curr Oncol Rep 5:108-113; Fried MW. 2002. Side effects of therapy of hepatitis C and their management. Hepatology 36:S237-S244; Wedemeyer 2002, Herrine SK. 2002. Approach to the patient with chronic hepatitis C virus infection. Ann Intern Med 136:747-757; Lauer GM, Walker BD. 2001. Hepatitis C virus infection. N Engl J Med 345:41-52; Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. 2001. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 132:296-305). These side effects include an influenza-like illness with fever, chills, myalgias and malaise in up to 82% of patients studied, with neuropsychiatric

complications such as depression, irritability and depression and anxiety in ~20% of patients. Bone marrow suppression with granulocytopenia, anemia or thrombocytopenia occurs in ~5%, as does alopecia. These side effects are frequently so severe that further treatment with IFN alpha is discontinued, thus further limiting the utility of IFN therapy. Therefore, new treatments for HCV are needed.

[0020] HIV therapy

[0021] Several drugs have been approved for treatment of HIV, including azidovudine (AZT), didanosine (dideoxyinosine, ddI), d4T, zalcitabine (dideoxycytosine, ddC), nevirapine, lamivudine (epivir, 3TC), saquinavir (Invirase), ritonavir (Norvir), indinavir (Crixivan), and delavirdine (Rescriptor). See M. I. Johnston & D. F. Hoth, Science, 260(5112), 1286-1293 (1993) and D. D. Richman, Science, 272(5270), 1886-1888 (1996). An alternative treatment for HCV has been ribavirin. Ribavirin is an anti-viral with a broad range of target viral activities. Ribavirin is a guanosine analogue harboring a modified base (1-β-D-ribo-furanosyl- -1,2,4-trizole-3-carboxamide), and has been proposed to inhibit the cellular enzyme inosine monophosphate dehydrogenase, resulting in a decrease of guanosine triphosphate. Damen, M., and Bresters, D., in H. W. (ed.): Curr. Stud. Hematol. Blood Transf., Darger Publishers 1998, Basel. However, ribavirin will cause side effects. Christie, J. M. and Chapman, R. W., Hosp Med. 60, 357 (1999). In particular ribavirin accumulates in the erythrocytes of patients and can cause hemolytic anemia.

[0022] An AIDS vaccine (Salk's vaccine) has been tested and several proteins which are chemokines from CD8 have been discovered to act as HIV suppressors. In addition to the above synthetic nucleoside analogs, proteins, and antibodies, several plants and substances derived from plants have been found to have *in vitro* anti-HIV activity. However, HIV virus is not easily destroyed nor is there a good mechanism for keeping the host cells from replicating the virus.

[0023] *In vitro* Use of Arginine Deprivation

[0024] Many studies over the last 30 years have demonstrated that extracellular arginine is required for viral replication *in vitro*. Historically this has been accomplished by making tissue culture media deficient in arginine and dialyzing the serum used as a supplement in order to achieve arginine free medium. Using this methodology to achieve arginine deprivation results in inhibition of replication of a large number of diverse families of viruses including: adeno virus (Rouse HC, Bonifas VH, Schlesinger RW. 1963. Dependence of adenovirus replication on arginine and inhibition of plaque formation by pleuropneumonia-like organisms. Virology

20:357-365), herpes virus (Tankersley RW Jr. 1964. Amino acid requirements of herpes simplex virus in human cells. J Bacteriol 87:608-613), SV 40 (Goldblum N, Ravid Z, Becker Y. 1968.Effect of withdrawal of arginine and other amino acids on the synthesis of tumour and viral antigens of SV40 virus. J Gen Virol 3:143-146), cytomegalovirus (Minamishima Y, Benyesh-Melnick M. 1969. Arginine-dependent events in cytomegalovirus infection. Bacteriol Proc 170:334-339), respiratory syncytial virus (Levine S, Buthala D, Hamilton RD. 1971. Late stage synchronization of respiratory syncytial virus replication. Virology 45:390-400), polyoma virus (Winters AL, Consigli RA, Rogers OR 1972. A non-functional arginine biosynthetic pathway in polyoma-infected mouse embryo cells. Biochem Biophys Res Comm 47:1045-1051), Newcastle disease virus (IInuma M, Maemo K, Matsumoto T. 1973. Studies on the assembly of Newcastle disease virus: an arginine-dependent step in virus replication. Virology 51:205-215), measles virus (Romano N, Scarlata G. 1973. Amino acid requirements of measles virus in HeLa cells. Arch Gesamte Virus Forschung 43:359-366), influenza (Lisok TP, Sominina AA. 1977. Improved methods of influenza virus propagation. I. Enhancement of virus reproduction in cell cultures. Acta Virol 21:234-240), and perhaps even more relevant, vaccinia virus (Holterman OA. 1969. Amino acid requirements for the propagation of vaccinia virus in Earle's L cells. J Gen Virol 4:585-591, Singer SH, Fitzgerald EA, Barile MF, Kirschstein RL. 1970. Effect of mycoplasmas on vaccinia virus growth: requirement of arginine. Proc Soc Exp Biol Med 133:1439-1442, Obert G, Tripier F, Guir J. 1971. Arginine requirement for late mRNA transcription of vaccinia virus in KB cells. Biochem Biophys Res Comm 44:362-367, Archard LC, Williamson JD. 1971. The effect of arginine deprivation on the replication of vaccinia virus. J Gen Virol 12:249-258.) and rabbit pox virus (Cooke BC, Williamson JD. 1973. Enhanced utilization of citrulline in rabbitpox virus-infected mouse sarcoma 180 cells. J Gen Virol 21:339-348). Vaccinia virus is the prototypical member of the Orthopoxvirus genera that includes smallpox (variola virus). Inhibition of viral replication is observed *in vitro*, even though protein synthesis and replication of infected cells is not affected.

[0025] Enzymes which degrade arginine are known and include arginine deiminase (ADI). However, a problem associated with the therapeutic use of such a heterologous protein is its antigenicity. The chemical modification of arginine deiminase from Mycoplasma arginini, via a cyanuric chloride linking group, with polyethylene glycol was described by Takaku, H, Misawa, S, Hayashi H and Miyazaki K. (1993). Chemical modification by polyethylene glycol of the anti-tumor enzyme arginine deiminase from *Mycoplasma arginini*. Jpn. J. Cancer Res.

84:1195-1200. However, the modified protein was toxic when metabolized due to the release of cyanide from the cyanuric chloride linking group.

[0026] There is a need for methods for inhibiting viral replication which do not have the problems associated with the prior art. The present invention is directed to these, as well as other, important ends.

[0027] SUMMARY OF THE INVENTION

[0028] The present invention is directed to methods of modulating viral replication comprising administering to a patient arginine deiminase bonded to polyethylene glycol. The present invention is also directed to methods of concurrently modulating viral replication and treating cancer, including, for example, sarcomas, hepatomas and melanomas. The present invention is also directed to methods of determining the susceptibility of an individual to arginine deprivation therapy for a viral infection, methods for improving liver function, and the like. These and other aspects of the present invention will be elucidated in the following detailed description of the invention.

[0029] DETAILED DESCRIPTION OF THE INVENTION

[0030] Overview

[0031] The present invention is based on the unexpected discovery that ADI modified with polyethylene glycol inhibits viral replication. ADI may be covalently bonded to polyethylene glycol with or without a linking group, although some embodiments utilize a linking group. PEG-20,000, for example, exhibits useful enzymatic activity levels, antigenicity, circulating half-life, efficacy, and relative ease of manufacture.

[0032] The mechanism by which lowering of extracellular arginine inhibits viral replication is not known. Herbivores such as human and mice (unlike carnivores which have an absolute requirement for arginine) (for review see Rodgers QR. 1994. Species variation in arginine requirements. In Proceedings from a Symposium Honoring Willard J. Visek - from Ammonia to Cancer and Gene Expression. Special Publication 86 - April 1994, Agriculture Experiment Station, University of Illinois, 211 Mumford Hall, Urbana, IL 61801, pp. 9-21) and most cells do not require arginine for growth as it may be synthesized from citrulline using two intracellular enzymes (argininosuccinate synthase and argininosuccinate lyase). Thus elimination of extracellular arginine dose not affect intracellular levels of arginine provided citrulline is

available to the cells. As viral replication is an intracellular process, it is unexpected that a decrease in extracellular arginine could inhibit viral replication.

[0033] Although not wishing to be bound by theory, one possible mechanism by which lowering of extracellular arginine may inhibit viral replication is by inhibiting nitric oxide synthesis. Nitric oxide is synthesized from extracellular arginine, thus elimination of this arginine pool effectively inhibits the production of this important metabolite. Although nitric oxide is thought to be protective against some virus infections (Akaike T, Maeda H. 2000. Nitric oxide and virus infection. Immunology 101:300-308), inhibition of nitric oxide synthesis has been shown to block the replication of lymphocytic choriomeningitis virus (Campbell IL Samimi A, Chiang CS. 1994. Expression of the inducible nitric oxide synthase. Correlation with neuropathology and clinical features in mice with lymphocytic choriomeningitis. J Immunol 153:3622-3629) and HIV (Blond D, Raoul H, LeGrand R, Dormont D. 2000. Nitric oxide synthesis enhances human immunodeficiency virus replication in primary human macrophages. J Virol 74:8904-8912). Inhibition of nitric oxide synthesis has also been shown to protect animals from the lethal effects of influenza (Akaike T, Noguchi Y, Ijiri S, Setoguchi K, Suga M, Zheng YM, Dietzschold B, Maeda H. 1996. Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. Proc Natl Acad Sci USA 93:2448-2453; Karupiah G, Chen J-H, Mahalingam S, Nathan CF, MacMicking JD. 1998. Rapid interferon γ-dependent clearance of influenza A virus and protection from consolidating pneumonitis in nitric oxide synthase 2-deficient mice. J Exp Med 188:1541-1546), polio virus (López-Guerrero JA, Carrasco L. 1998. Effect of nitric oxide on poliovirus infection of two human cell lines. J Virol 72:2538-2540), rabies virus (Ubol S, Sukwattanapan C, Maneerat Y. 2001. Inducible nitric oxide synthase delays death of rabies virus-infected mice. J Med Microbiol 50:238-42) and flavivirus (Kreil TR, Eibl MM. 1996. Nitric oxide and viral infection: no antiviral activity against a flavivirus *in vitro*, and evidence for contribution to pathogenesis in experimental infection *in vivo*. Virology 219:304-306). However, these previously used nitric oxide synthesis inhibitors have been limited by their toxicities (liver failure, seizure and death) in both animals and humans. Thus it is not clear that inhibition of viral replication resulting from elimination of arginine from the culture media (a process which clearly eliminates nitric oxide production) is the only mechanism by which inhibition of viral replication occurs. This stimulation/inhibition duality of nitric oxide and virus infection is also observed with nitric oxide in other pathological events (Colasanti M, Suzuki H. 2000. The dual

personality of NO. Trends Pharm Sci 21:249-252). Thus inhibition of nitric oxide should not be expected to abrogate all sequella of an infectious event (Bogdan C. 2001. Nitric oxide and the immune system. Nature Immunology 2:907-916). However, unlike the nitric oxide synthesis inhibitors used in the past, ADI-PEG 20 appears to be safe and effective in inhibiting production of nitric oxide and can be used to help elucidate the role of this biomediator in protection against viral infection.

[0034] Definitions

[0035] Throughout the present disclosure, the following abbreviations may be used: PEG, polyethylene glycol; ADI, arginine deiminase; SS, succinimidyl succinate; SSA, succinimidyl succinamide; SPA, succinimidyl propionate; and NHS, N-hydroxy-succinimide.

[0036] ADI covalently modified with polyethylene glycol (with or without a linking group) may be hereinafter referred to as "ADI-PEG", or "PEG-ADI".

[0037] "Polyethylene glycol" or "PEG" refers to mixtures of condensation polymers of ethylene oxide and water, in a branched or straight chain, represented by the general formula $H(OCH2CH2)_nOH$, wherein n is at least 4. "Polyethylene glycol" or "PEG" is used in combination with a numeric suffix to indicate the approximate weight average molecular weight thereof. For example, PEG-5,000 (PEG5) refers to polyethylene glycol molecules having an average molecular weight of about 5,000; PEG-12,000 (PEG12) refers to polyethylene glycol molecules having an average molecular weight of about 12,000; and PEG-20,000 (PEG20) refers to polyethylene glycol molecules having an average molecular weight of about 20,000.

[0038] As used herein, the term "individual" refers to an animal, in some embodiments a mammal, and in some embodiments a human. The term "individual" includes biological samples taken from such animals.

[0039] As used herein, the term "viral disease" refers to diseases and disorders caused by a virus. Viral diseases include without limitation viruses that infect animals or mammals, including humans. Human viruses include viruses from the following viral families: Pox, Herpes, Adeno, Papova, Parvo, Hepadna, Picorna, Calici, Astro, Toga, Flavi, Corona, Paramyxo, Orthomyxo, Bunya, Arena, Rhabdo, Filo, Borna, Reo, and Retro.

[0040] Examples of viruses and associated diseases that may be treated by the present invention include without limitation: variola (smallpox); herpesviruses, such as herpes simplex virus (cold sores), varicella-zoster (chicken pox, shingles), Epstein-Barr virus (mononucleosis, Burkitt's lymphoma), KSHV (Kaposi's sarcoma), and cytomegalovirus (blindness); adenoviruses;

hepatitis (A/B/C); polioviruses, rhinociruses, rubella, yellow fever, West Nile virus, dengue, equine encephalitis, respiratory syncytial virus (RSV), parainfluenza virus, and tobacco mosaic virus.

[0041] In some embodiments the virus is one or more of HIV, influenza, polio viruses, herpes simplex, hepatitis B, hepatitis C and other viral strains of hepatitis, Kaposi's sarcoma, rhinoviruses, West Nile virus, smallpox, and vaccinia, among others.

[0042] As used herein, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In some embodiments of the present invention, inhibition is the form of modulation of gene expression.

[0043] As used herein, the term "inhibit" refers to a reduction or decrease in a quality or quantity, compared to a baseline. For example, in the context of the present invention, inhibition of viral replication refers to a decrease in viral replication as compared to baseline. In some embodiments there is a reduction of about 30%, about 40%, about 50%, about 60%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%, and about 100%. Those of ordinary skill in the art can readily determine whether or not viral replication has been inhibited and to what extent.

[0044] As used herein, the term "about" refers to +/- 20%, +/- 15%, +/- 10%, or +/- 5% of the value.

[0045] As used herein, the term "biocompatible" refers to materials or compounds which are generally not injurious to biological functions and which will not result in any degree of unacceptable toxicity, including allergenic and disease states.

[0046] "Circulating half life" refers to the period of time, after injection of the modified ADI into a patient, until a quantity of the ADI has been cleared to levels one half of the original peak serum level. Circulating half-life may be determined in any relevant species, including humans or mice.

[0047] As used herein, the terms "covalently bonded", "bonded" and "coupled" are used interchangeably and refer to a covalent bond linking ADI to the PEG molecule, either directly or through a linker.

As used herein, the term "therapeutically effective amount" is meant an amount of a compound of the present invention effective to yield the desired therapeutic response. The specific therapeutically effective amount will, obviously, vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal or animal

being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives. In the context of improving liver function, the term "therapeutically effective amount" refers to an amount of arginine deiminase bonded to polyethylene glycol that improves liver function. In some embodiments the therapeutically effective amount is effective to improve the Child-Pugh scale or the Mayo End-stage Liver Disease (MELD) score of the individual. In some embodiments the therapeutically effective amount is effective to improve liver function based on comparison of markers of liver function including, without limitation, billrubin levels, creatine levels, and international normalized ratio.

[0048] As used herein, the term "an amount effective to inhibit viral replication" refers to the amount of a compound comprising ADI covalently bonded via a linking group to polyethylene glycol administered to an individual that results in a reduced level of viral replication and thus a reduced amount of detectable virus in the individual, *i.e.*, a reduction in viral titer or viral load. To determine an amount effective to inhibit viral replication, the individual's viral load can be determined prior to treatment with a compound of the present invention and then subsequent to treatment. The level of viral replication can be quantified by any number of routine methodologies including, for example: quantifying the actual number of viral particles in a sample prior to and subsequent to compound administration, and quantifying the level of one or more viral antigen present in a sample prior to and subsequent to compound administration. In some embodiments "an amount effective to inhibit viral replication" is the amount necessary to decrease plasma arginine concentrations below about 5 μM. Methods of measuring plasma arginine concentrations are well known in the art.

[0049] Assays for viral replication also provide one with the ability to determine the efficacy of viral inhibitors and are well known to those skilled in the art. Such assays may be conducted *in vivo* or *in vitro*. HCV is known to occur in chimpanzees where the infection closely resembles that seen in humans. There have also been reports of experimental infection in tupaias, closely related to the primates, and in immunodeficient mice. (Xie, Z. C. et al., Virology, 244, 513 (1998); Schinazi, R. F. et al., Antiviral Chem. Chemother. 10, 99, (1999)).

[0050] The inhibition of viral replication contributes to a reduction in the severity of the viral infection or of the symptoms of the viral infection.

[0051] As used herein, the term "prophylactically effective amount" is meant an amount of a compound of the present invention effective to yield the desired prophylactic response. The

specific prophylactically effective amount will, obviously, vary with such factors as the particular virus, the physical condition of the patient, the type of mammal or animal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives

[0052] As used herein "combination therapy" means that the individual in need of treatment is given another drug for the disease in conjunction with PEG-ADI. This combination therapy can be sequential therapy where the individual is treated first with one or more drugs and then the other, or two or more drugs are given simultaneously.

[0053] As used herein, the phrase "arginine deprivation therapy" refers to a treatment regimen that involves the use of an agent that reduces, minimizes, or abolishes arginine levels in the patient. Arginine deprivation therapy is often performed using ADI. Arginine deprivation therapy and agents used in arginine deprivation therapy are described in detail in allowed U.S. application Ser. No. 09/023,809, filed February 13, 1998, now U.S. Patent 6,183,738, issued February 6, 2001; and pending application U.S. Ser. No. 09/504, 280, filed February 15, 2000, each of which is hereby incorporated by reference in its entirety.

[0054] As used herein, the term "an individual suspected of having been exposed to one or more viruses" refers to an individual who has not been diagnosed as being positive for one or more viruses but who could possibly have been exposed to one or more viruses due to a recent high risk activity or activity that likely put them in contact with the viruses. For example, an individual suspected of having been exposed to HIV refers to an individual that has been stuck with a needle that has been in contact with either a sample that contains HIV or HIV infected individual. Examples of such samples include, without limitation, laboratory or research samples or samples of blood, semen, bodily secretions, and the like from patients. Individuals suspected of being exposed to HCV include individuals that have received blood transfusions with blood of unknown quality. The blood that is being transfused may have not been tested or the test results indicating that the blood does not contain HCV are not reliable or are doubted. In some embodiments, an individual suspected of being infected with a virus includes individuals who have been exposed to the virus through another individual including, for example, through sexual intercourse, contact with bodily fluids of another individual, sharing of hypodermic needles, and the like. The individual from which the virus originated may or may not have been tested for the presence and/or absence of the virus. The term "an individual suspected of having been exposed to one or more viruses" also includes individuals who have

been diagnosed as being positive for one virus but are also infected with at least one further virus. For example, often those infected with HIV are also positive for one or more forms of hepatitis. Such an individual may be classified as being at "high-risk" for one or more viruses.

[0055] As used herein, the term "selectively inhibit" refers selective inhibition of viral replication and is, in some embodiments, the ratio of CC_{50}/EC_{50} % of viral mRNA levels. An SI>10 is considered to reflect a selective inhibition of viral replication.

[0056] As used herein, the term "sample" refers to biological material from a patient. The sample assayed by the present invention is not limited to any particular type. Samples include, as non-limiting examples, single cells, multiple cells, tissues, tumors, biological fluids, biological molecules, or supernatants or extracts of any of the foregoing. Examples include tissue removed for biopsy, tissue removed during resection, blood, urine, lymph tissue, lymph fluid, cerebrospinal fluid, mucous, and stool samples. The sample used will vary based on the assay format, the detection method and the nature of the tumors, tissues, cells or extracts to be assayed. Methods for preparing samples are well known in the art and can be readily adapted in order to obtain a sample that is compatible with the method utilized.

[0057] **ADI**

[0058] Arginine deiminase catalyzes the conversion of arginine to citrulline, and may be used to eliminate arginine. In the present invention, the arginine deiminase gene may be derived, cloned or produced from any source, including, for example, microorganisms, recombinant biotechnology or any combination thereof. Arginine deiminase may be cloned from microorganisms of the genus *Mycoplasma*. In some embodiments, the arginine deiminase is cloned from *Mycoplasma arginini, Mycoplasma hominus, Mycoplasma arthritides*, or any combination thereof. In some embodiments, the arginine deiminase used in the present invention may have one or more of the amino acid sequences of SEQ ID NOS: 1-10 and 13-21.

[0059] Native arginine deiminase may be found in microorganisms and is antigenic and rapidly cleared from circulation in a patient. These problems may be overcome by covalently modifying arginine deiminase with polyethylene glycol (PEG). Arginine deiminase covalently modified with polyethylene glycol (with or without a linking group) may be hereinafter referred to as "ADI-PEG." When compared to native arginine deiminase, ADI-PEG retains most of its enzymatic activity, is far less antigenic, has a greatly extended circulating half-life, and is much more efficacious in the treatment of tumors.

[0060] Certain disadvantages have come to be associated with the isolation of arginine

deiminase from organisms. Although effective in killing tumor cells *in vitro*, arginine deiminase isolated from *Pseudomonas pudita* failed to exhibit efficacy *in vivo* because it had little enzyme activity at a neutral pH and was rapidly cleared from the circulation of experimental animals. Arginine deiminase derived from *Mycoplasma arginini* (SEQ ID NO:5) is described, for example, by Takaku H, Takase M, Abe S, Hayashi H and Miyazaki K. (1992). *In vivo* anti-tumor activity of arginine deiminase purified from *Mycoplasma arginini*. Int. J. Cancer 51:244-249, and U.S. Patent No. 5,474,928, the disclosures of which are hereby incorporated by reference herein in their entirety. A problem associated with the therapeutic use of such a heterologous protein is its antigenicity. The chemical modification of arginine deiminase from *Mycoplasma arginini*, via a cyanuric chloride linking group, with polyethylene glycol was described by Takaku, H, Misawa, S, Hayashi H and Miyazaki K. (1993). Chemical modification by polyethylene glycol of the anti-tumor enzyme arginine deiminase from *Mycoplasma arginini*. Jpn. J. Cancer Res. 84:1195-1200. The modified protein was toxic when metabolized due to the release of cyanide from the cyanuric chloride linking group.

[0061] The production of arginine deiminase via recombinant DNA techniques also provides for certain disadvantages. For example, arginine deiminase produced in *Escherichia coli* is enzymatically inactive and thus must be denatured and then properly renatured in order for it to become enzymatically active. The usual method for renaturing arginine deiminase produced in *E. coli* is to isolate the inactive enzyme, dissolve it in guanidinium hydrochloride and renature it by rapid dilution into low ionic strength buffer. This last step requires very large volumes of buffer thus making the manufacture of arginine deiminase both expensive and time consuming. However, recombinant technology does have certain advantages. For example, organisms more amenable to fermentation can be used as hosts. Additionally, these fermentation hosts are generally much less pathogenic and larger amounts of arginine deiminase can be obtained. It has been shown the *E. coli* may produce large amounts of *Mycoplasma* arginine deiminase.

[0062] Chemical and genetic modification of the arginine deiminase enzyme can affect its biological activities. For example, it has been shown that arginine deiminase is typically antigenic and rapidly cleared from circulation in a patient. However, it has also been shown that the formulation of arginine deiminase with polyethylene glycol reduces the antigenicity and increases the circulating half-life of the enzyme. Abuchowski et al., *Cancer Biochem. Biophys.* 7:175-186 (1984); Abuchowski et al., *J. Biol. Chem.* 252:3582-3586 (1977). In particular,

arginine deiminase can be covalently modified with polyethylene glycol. Arginine deiminase covalently modified with polyethylene glycol (with or without a linking group) may be hereinafter referred to as "ADI-PEG." In U.S. Patent Application Serial No. 09/023,809, Clark describes improved modifications of arginine deiminase from *Mycoplasma hominus* (SEQ ID NO:1), *Mycoplasma arginini* (SEQ ID NO:5), and *Mycoplasma arthritides* (SEQ ID NO:7) with polyethylene glycol, the disclosure of which is hereby incorporated by reference herein in its entirety. When compared to native arginine deiminase, ADI-PEG retains most of its enzymatic activity, is far less antigenic, has a greatly extended circulating half-life, and is much more efficacious in the treatment of tumors. For purposes of the invention, the modification of any arginine deiminase with polyethylene glycol may be referred to as pegylation.

[0063] It is to be understood that arginine deiminase derived from other organisms may also have pegylation sites corresponding to 112 position of arginine deiminase from *Mycoplasma hominus*. For example, arginine deiminase from *Streptococcus pyrogenes* has lysine at the 104 position, arginine deiminase from *Mycoplasma pneumoniae* has lysine at the 106 position, and arginine deiminase from *Qiardia intestinalis* has lysine at the 114 position. In addition, arginine deiminase from some organisms may have lysines corresponding to the same general location as the 112 position of arginine deiminase from *Mycoplasma hominus*. The location of lysine in arginine deiminase from such organisms may be indicated as follows:

Table 1: Pegylation sites of arginine deiminase from various organisms

Organisms producing arginine deiminase	**Position of lysine in arginine deiminase**
Mycoplasma hominus (SEQ ID NO:1)	112
Mycoplasma arginini (SEQ ID NO:5)	111
Clostridium perfringens(SEQ ID NO:18)	105
Bacillus licheniformis(SEQ ID NO:19)	97, 108
Borrelia burgdorferi(SEQ ID NO:15)	102, 111
Borrelia afzellii (SEQ ID NO:16)	101
Enterococcus faecalis(SEQ ID NO:20)	102, 110
Streptococcus pyogenes(SEQ ID NO:13)	104
Streptococcus pneumoniae(SEQ ID NO:14)	103
Lactobacillus sake (SEQ ID NO:21)	97, 106

Qiardia intestinalis(SEQ ID NO:17)	114, 116

[0064] It is presently believed that the attachment of polyethylene glycol to such lysines or combinations thereof may inactivate the enzyme. It is presently believed that amino acid substitutions at such lysines may result in a protein that loses less of its enzymatic activity upon pegylation.

[0065] In some embodiments the present invention provides for certain amino acid substitutions in the polypeptide chain of arginine deiminase. These amino acid substitutions provide for modified arginine deiminase that loses less activity upon pegylation; i.e. upon pegylation, the reduction of enzyme activity following pegylation in the modified arginine deiminases is less than the reduction of enzyme activity following pegylation in the unmodified arginine deiminases. By eliminating pegylation sites at or adjacent to the catalytic region of enzyme, optimal pegylation can be achieved without the traditional loss of activity. As discussed above, arginine deiminase from certain organisms have pegylation sites located at various positions on the peptide chain. While not limiting the present invention, it is presently believed that arginine deiminase may have the amino acid lysine located at or adjacent to the catalytic region of the enzyme and that pegylation of these sites may inactivate the enzyme. By eliminating at least one of these pegylation sites, pegylation can be achieved and more enzyme activity retained. In accordance with the invention, in some embodiments lysine is substituted with glutamic acid, valine, aspartic acid, alanine, isoleucine, leucine or combinations thereof. In some embodiments lysine is substituted with glutamic acid. In some embodiments of the invention, modified arginine deiminase from *Mycoplasma hominus* has an amino acid substitution at Lys^{112}, Lys^{374}, Lys^{405}, Lys^{408} or combinations or subcombinations thereof. In some embodiments modified arginine deiminase from *Mycoplasma hominus* has an amino acid substitution Lys^{112} to Glu^{112}, Lys^{374} to Glu^{374}, Lys^{405} to Glu^{405}, Lys^{408} to Glu^{408} or combinations thereof. In some embodiments modified arginine deiminase from *Mycoplasma hominus* has lysine at position 112 substituted with glutamic acid (SEQ ID NO:2).

[0066] The present invention thus provides for certain amino acid substitutions in the polypeptide chain of arginine deiminase. Such amino acid substitutions can eliminate the problematic structural characteristics in the peptide chain of arginine deiminase. Such amino acid substitutions provide for improved renaturation of the modified arginine deiminase. These

amino acid substitutions make possible rapid renaturing of modified arginine deiminase using reduced amounts of buffer. These amino acid substitutions may also provide for increased yields of renatured modified arginine deiminase. In some embodiments of the invention, the modified arginine deiminase has a single amino acid substitution at Pro^{210}. As mentioned above, arginine deiminase derived from *Mycoplasma hominus* has the amino acid proline located at the 210 position. While not limiting the present invention, it is presently believed that the presence of the amino acid proline at position 210 results in a bend or kink in the normal polypeptide chain that increases the difficulty of renaturing (i.e., refolding) arginine deiminase. Substitutions for proline at position 210 may make possible the rapid renaturation of modified arginine deiminase using reduced amounts of buffer. Substitutions for proline at position 210 may also provide for increased yields of renatured modified arginine deiminase. In some embodiments, the proline at position 210 is substituted with serine (SEQ ID NO:3). It is to be understood that in accordance with this aspect of the invention, other substitutions at position 210 may be made. Examples of substitutions include Pro^{210} to Thr^{210}, Pro^{210} to Arg^{210}, Pro^{210} to Asn^{210}, Pro^{210} to Gln^{210} or Pro^{210} to Met^{210}. By eliminating those structural characteristics associated with the amino acid of position 210 of the wild-type arginine deiminase, proper refolding of the enzyme can be achieved.

[0067] In some embodiments of the invention, the modified arginine deiminase has multiple amino acid substitutions. The modified arginine deiminase may have at least one amino acid substitution eliminating pegylation sites at or adjacent a catalytic region of the enzyme. The modified arginine deiminase may also have at least one amino acid substitution eliminating those structural characteristics that interfere with the renaturation of the enzyme. The amino acid substitutions may thus provide for a modified arginine deiminase of the invention. The amino acid substitutions may provide for the pegylation of modified arginine deiminase without a loss of enzymatic activity. The amino acid substitutions may provide for a modified arginine deiminase that can be rapidly renatured using reduced amounts of buffer. The amino acid substitutions may also provide for increased yields of renatured modified arginine deiminase. In some embodiments, the modified arginine deiminase derived from *Mycoplasma hominus* includes the proline at position 210 substituted with serine and the lysine at position 112 substituted with glutamic acid (SEQ ID NO:4). As discussed above, however, it is to be understood that the modified arginine deiminase may include other substitutions. In some embodiments, conservative substitutions may be made at positions 112 and/or 210 of the wild-

type arginine deiminase.

[0068] Modified arginine deiminase was expressed in JM101 cells as previously described by Takaku et al., *supra*. The modified arginine deiminase included glutamic acid at the 112 position and serine at the 210 position. In some embodiments the amino acid sequence of modified arginine deiminase from *Mycoplasma hominus* is a sequence of SEQ ID NO:4.

[0069] In some embodiments arginine deiminase is derived from *Mycoplasma hominus*, *Mycoplasma pneumoniae*, *Mycoplasma arginini*, *Qiardia intestinalis*, *Clostridium perfringens*, *Bacillus licheniformis*, *Borrelia burgdorferi*, *Borrelia afzellii*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Lactobacillus sake* or *Qiardia intestinalis* arginine deiminase.

[0070] In some embodiments arginine deiminase is derived from *Mycoplasma hominus* arginine deiminase (SEQ ID NO:1). In some embodiments, the arginine deiminase comprises at the substitution or deletion of at least one proline residue as compared to SEQ ID NO:1. In some embodiments, the substitution or deletion of at least one proline residue comprises substitution or deletion of the proline residue at or corresponding to residue 210 of SEQ ID NO:1. In some embodiments, the substitution or deletion of at least one proline residue comprises substitution of the proline residue at or corresponding to residue 210 of SEQ ID NO:1 with Ser, Thr, Arg, Asn, Gln, or Met. In some embodiments, the substitution or deletion of at least one proline residue comprises substitution of the proline residue at or corresponding to residue 210 of SEQ ID NO:1 with Ser.

[0071] In some embodiments of the present invention the arginine deiminase is modified and comprises at least one amino acid substitution or deletion wherein the modified arginine deiminase has a reduced number of pegylation sites at or adjacent to a catalytic region, as compared to SEQ ID NO:1. In some embodiments, the substitution or deletion of at least one lysine residue comprises the substitution or deletion of at least one lysine residue at or corresponding to residues 112, 374, 405 or 408 of SEQ ID NO:1. In some embodiments, the substitution or deletion of at least one lysine residue comprises the substitution of at least one lysine residue at or corresponding to residues 112, 374, 405 or 408 of SEQ ID NO:1 with Glu, Val, Asp, Ala, Ile or Leu. In some embodiments, the substitution or deletion of at least one lysine residue comprises substitution of the lysine residue at or corresponding to residue 112 of SEQ ID NO:1 with Glu, Val, Asp, Ala, Ile or Leu. In some embodiments, the substitution or deletion of at least one lysine residue comprises substitution of the lysine residue at or

corresponding to residue 112 of SEQ ID NO:1 with Glu. In some embodiments, the modified arginine deiminase comprises the further substitution or deletion of at least one proline residue.

[0072] In some embodiments, the substitution or deletion of at least one proline residue comprises substitution of the proline residue at or corresponding to residue 210 of SEQ ID NO:1 with Ser, Thr, Arg, Asn, Gln, or Met.

[0073] In some embodiments the arginine deiminase comprises arginine deiminase modified to be free of at least one pegylation site at or adjacent to a catalytic region as compared to SEQ ID NO:1, wherein said modified arginine deiminase comprises at least one amino acid substitution or deletion at or corresponding to residues 112, 374, 405, or 408 of SEQ ID NO:1. In some embodiments the at least one amino acid substitution or deletion comprises substitution of the lysine residue at or corresponding to residue 112 of SEQ ID NO:1 with Glu, Val, Asp, Ala, Ile or Leu. In some embodiments the at least one amino acid substitution or deletion further comprises substitution or deletion of at least one proline residue. In some embodiments the substitution or deletion of at least one proline residue comprises substitution or deletion of the proline residue at or corresponding to residue 210 of SEQ ID NO:1. In some embodiments the substitution or deletion of at least one proline residue comprises substitution of the proline residue at or corresponding to residue 210 of SEQ ID NO:1 with Ser, Thr, Arg, Asn, Gln, or Met.

[0074] In some embodiments the arginine deiminase from *Mycoplasma hominus* comprises a substitution of lysine at residue 112 of SEQ ID NO:1 with glutamic acid (SEQ ID NO:2). In some embodiments the arginine deiminase from *Mycoplasma hominus* comprises a substitution of proline at residue 210 of SEQ ID NO:1 with serine (SEQ ID NO:3). In some embodiments the arginine deiminase from *Mycoplasma hominus* comprises a substitution of lysine at residue 112 of SEQ ID NO:1 with glutamic acid and a substitution of proline at residue 210 of SEQ ID NO:1 with serine (SEQ ID NO:4). In some embodiments arginine deiminase from *Mycoplasma arginini* comprises a substitution of lysine at residue 111 of SEQ ID NO:5 with glutamic acid (SEQ ID NO:6). In some embodiments the arginine deiminase from *Mycoplasma arthritides* comprises substitutions of lysine at residues 111 and 112 of SEQ ID NO:7 with glutamic acid (SEQ ID NO:8). In some embodiments the arginine deiminase from *Mycoplasma arthritides* comprises a substitution of lysine at residue 111 of SEQ ID NO:7 with glutamic acid (SEQ ID NO:9). In some embodiments the arginine deiminase from *Mycoplasma arthritides* comprises a substitution of lysine at residue 112 of SEQ ID NO:7 with glutamic acid (SEQ ID NO:10).

[0075] Such modifications and/or substitutions as well as nucleotide and polypeptide sequences are described in U.S. Patent No. 6,183,738, issued February 6, 2001, and co-pending Application Ser. No. 09/564,559, filed May 4, 2000, each of which is hereby incorporated by reference in its entirety.

[0076] Polyethylene Glycol

[0077] There are many polyethylene glycols available that differ in their molecular weight and linking group. These PEGs can have varying effects on the antigencity, immunogenicity and circulating half-life of a protein (Zalipsky, S. and Lee, C. Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications. Pp. 347-370, Plenum Press, New York, 1992; Monfardini, C., *et. al.* bioconjugate Chem. 6, 62-69, 1995; Delgado C; Francis GE; Fisher D. The uses and properties of PEG-linked proteins. Crit. Rev. Ther. Drug Carrier Sys., 9:249-304, 1992.)

[0078] In some embodiments of the present invention, each polyethylene glycol molecule has an average molecular weight of about 10,000 to about 50,000; from about 12,000 to about 40,000, from about 15,000 to about 30,000; and about 20,000. Generally, polyethylene glycol with a molecular weight of 30,000 or more is difficult to dissolve, and yields of the formulated product are greatly reduced.

[0079] The polyethylene glycol may be a branched or straight chain. In some embodiments the polyethylene glycol is a straight chain. Increasing the molecular weight of the polyethylene glycol generally tends to decrease the immunogenicity of the ADI. The polyethylene glycols having the molecular weights described in the present invention may be used in conjunction with ADI, and, optionally, a biocompatible linking group, to treat viral diseases.

[0080] Pegylation

[0081] ADI may be covalently bonded to PEG via a biocompatible linking group, using methods known in the art, as described, for example, by Park et al, Anticancer Res., 1:373-376 (1981); and Zaplipsky and Lee, Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications, J.M. Harris, ed., Plenum Press, NY, Chapter 21 (1992), the disclosures of which are hereby incorporated by reference herein in their entirety.

[0082] The linking group used to covalently attach PEG to ADI may be any compatible linking group. In some embodiments the linking group is a biocompatible linking group. As discussed above, "biocompatible" indicates that the compound or group is non-toxic and may be utilized *in vitro* or *in vivo* without causing injury, sickness, disease or death. PEG can be

bonded to the linking group, for example, via an ether bond, an ester bond, a thiol bond or an amide bond. Suitable linking groups include, for example, an ester group, an amide group, an imide group, a carbamate group, a carboxyl group, a hydroxyl group, a carbohydrate, a succinimide group (including, for example, succinimidyl succinate (SS), succinimidyl propionate (SPA), succinimidyl carboxymethylate (SCM), succinimidyl succinamide (SSA) or N-hydroxy succinimide (NHS)), an epoxide group, an oxycarbonylimidazole group (including, for example, carbonyldimidazole (CDI)), a nitro phenyl group (including, for example, nitrophenyl carbonate (NPC) or trichlorophenyl carbonate (TPC)), a trysylate group, an aldehyde group, an isocyanate group, a vinylsulfone group, a tyrosine group, a cysteine group, a histidine group or a primary amine. In some embodiments the linking group is an ester group and/or a succinimide group. In some embodiments, the linking group is SS, SPA, SCM, SSA or NHS.

[0083] In the present invention, the particular linking groups do not appear to influence the circulating half-life of PEG-ADI or its specific enzyme activity. However, if a linking group is used, in some embodiments it is important to use a biocompatible linking group. The PEG which is attached to the protein may be either a single chain, as with SS-PEG, SPA-PEG and SC-PEG, or a branched chain of PEG may be used, as with PEG2-NHS.

[0084] Alternatively, ADI may be coupled directly to PEG (*i.e.*, without a linking group) through an amino group, a sulfhydral group, a hydroxyl group or a carboxyl group. In some embodiments, PEG is coupled to lysine residues on ADI.

[0085] **ADI-PEG**

[0086] The attachment of PEG to ADI increases the circulating half-life of ADI. The number of PEG molecules on ADI appears to be related to the circulating half-life of the enzyme, while the amount of retained enzymatic activity appears related to the average molecular weight of the PEG used. Increasing the number of PEG units on ADI decreases the enzymatic activity of the enzyme. Also, it is known that some PEG formulations are difficult to produce and yield relatively low amounts of product. Thus, to achieve an efficacious product, a balance needs to be achieved among circulating half-life, antigenicity, efficiency of production, and enzymatic activity.

[0087] Generally, PEG is attached to a primary amine of ADI. Selection of the attachment site of polyethylene glycol on the arginine deiminase is determined by the role of each of the sites within the active domain of the protein, as would be known to the skilled artisan. PEG may be attached to the primary amines of arginine deiminase without substantial loss of enzymatic

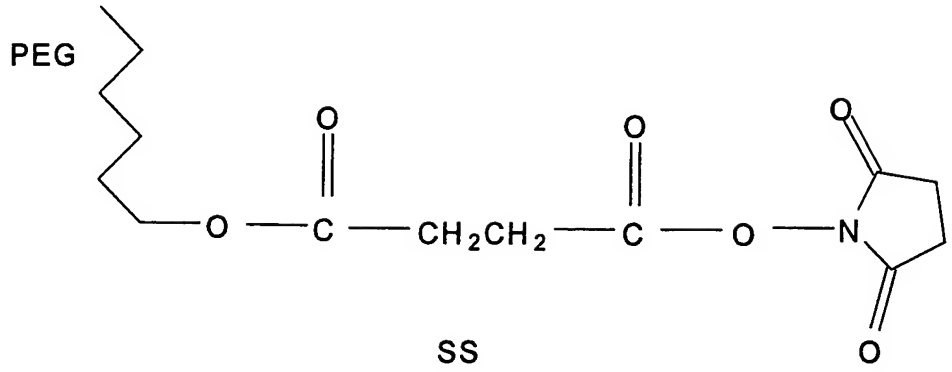
activity. For example, ADI cloned from *Mycoplasma arginini*, *Mycoplasma arthritides* and *Mycoplasma hominus* has about 17 lysines that may be modified by this procedure. In other words, the 17 lysines are all possible points at which ADI can be attached to PEG via a biocompatible linking group, such as SS, SPA, SCM, SSA and/or NHS. PEG may also be attached to other sites on ADI, as would be apparent to one skilled in the art in view of the present disclosure.

[0088] From 1 to about 30 PEG molecules may be covalently bonded to ADI. In some embodiments ADI is modified with about 7 to about 15 PEG molecules, from about 9 to about 12 PEG molecules. In other words, about 30% to about 70% of the primary amino groups in arginine deiminase are modified with PEG, about 40% to about 60%, about 45% to about 55%, and about 50% of the primary amino groups in arginine deiminase are modified with PEG. In some embodiments when PEG is covalently bonded to the end terminus of ADI, only 1 PEG molecule is utilized. Increasing the number of PEG units on ADI increases the circulating half life of the enzyme. However, increasing the number of PEG units on ADI decreases the specific activity of the enzyme. Thus, in some embodiments a balance needs to be achieved between the two, as would be apparent to one skilled in the art in view of the present disclosure.

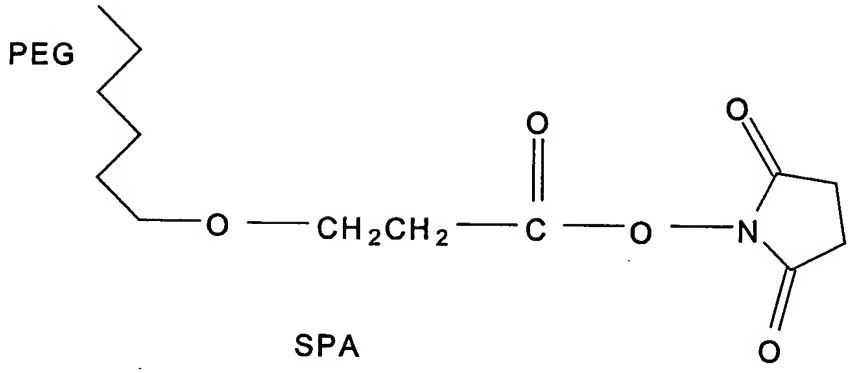
[0089] In the present invention, in some embodiments the linking groups attach to a primary amine of arginine deiminase via a maleimide group. Once coupled with arginine deiminase, SS-PEG has an ester linkage next to the PEG, which may render this site sensitive to serum esterase, which may release PEG from ADI in the body. SPA-PEG and PEG2-NHS do not have an ester linkage, so they are not sensitive to serum esterase.

[0090] The structural formulas of certain linking groups useful in the present invention are set forth below.

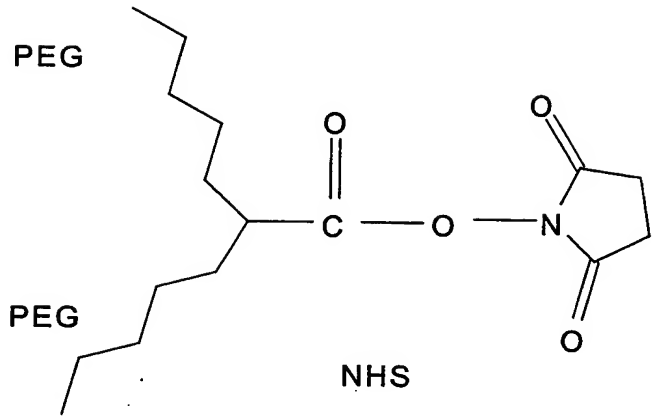
SS-PEG:



SPA-PEG:



PEG2-NHS



[0091] Methods of Treatment

[0092] In some embodiments, the present invention provides methods of inhibiting viral replication in an individual comprising administering to said individual a therapeutically or prophylactically effective amount of a compound comprising ADI covalently bonded via a linking group to polyethylene glycol, wherein each polyethylene glycol molecule has an average molecular weight of from about 10,000 to about 30,000. In some embodiments ADI is modified with polyethylene glycol molecules, each molecule having an average molecular weight of about 20,000. In some embodiments the linking group is selected from the group consisting of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group, a histidine group and combinations thereof. In some embodiments the linking group is succinimidyl succinate. In some embodiments from about 7 to about 15 polyethylene glycol molecules are bonded to arginine deiminase. In some embodiments from about 9 to about 12 polyethylene glycol molecules are bonded to arginine deiminase. In some embodiments the arginine deiminase is derived from a microorganism of the genus *Mycoplasma*. In some embodiments the arginine deiminase is derived from *Mycoplasma arginini*, *Mycoplasma hominus*, *Mycoplasma arthritides* and combinations thereof. In some embodiments the virus is HCV. In some embodiments the methods further comprise the step of administering a therapeutically effective amount of an additional anti-viral agent prior to, simultaneously with, or following administration of the arginine deiminase.

[0093] A therapeutically effective amount of one of the compounds of the present invention

is an amount that is effective to inhibit viral replication. Generally, treatment is initiated with small dosages which can be increased by small increments until the optimum effect under the circumstances is achieved. Generally, a therapeutic dosage of compounds of the present invention may be from about 1 to about 200 mg/kg twice a week to about once every two weeks. For example, the dosage may be about 1 mg/kg once a week as a 2 ml intravenous injection to about 20 mg/kg once every 3 days. The compounds can be administered in one dose, continuously or intermittently throughout the course of treatment. ADI-PEG maybe administered several times each day, once a day, once a week, or once every two weeks.

[0094] In some embodiments, ADI-PEG is administered in a weekly dose of at least about 40 IU/m^2, at least about 80 IU/m^2, at least about 160 IU/m^2, or at least about 200 IU/m^2. In some embodiments the dose administered lowers plasma levels of arginine to less than about 10, μM, 5 μM, 1 μM, or 100 nM. In some embodiments, ADI-PEG20 is administered in a weekly dose of about 160 IU/m^2 resulting in a plasma level in the patient of less than about 5 μM.

[0095] The present invention provides methods of inhibiting replication of one or more viruses in an individual comprising administering a therapeutically or prophylactically effective amount of an arginine deiminase bonded to polyethylene glycol to said individual. In some embodiments the virus is a human virus. In some embodiments the virus is HCV. In some embodiments, the individual is infected with two or more different viruses. In some embodiments the two or more viruses are HIV and HCV. In some embodiments the presence and and/or identity of an infecting virus is unknown at or before the time of administration. In some embodiments the methods further comprise the step of administering a therapeutically effective amount of an additional anti-viral agent prior to, simultaneously with, or following administration of the arginine deiminase.

[0096] The present invention also provides methods for treating an individual suspected of having been exposed to one or more viruses comprising administering a therapeutically or prophylactically effective amount of an arginine deiminase bonded to polyethylene glycol to said individual. As discussed above, some individuals who have not been diagnosed as being infected with one or more viruses are put in circumstances where it is possible that they could have possibly been exposed to the virus. The treatment of individuals suspected of being exposed to one or more viruses may also include the administration of additional therapeutics as described above. The course of prophylactic treatment may be performed in conjunction with periodic monitoring for indications of viral infection. In some embodiments, following

commencement of treatment according to the present invention the individual is diagnosed as being positive for one or more viruses.

[0097] In some embodiments the present invention provides methods of inhibiting viral replication in an individual at risk for one or more viruses. The methods comprise administering to the individual an amount of a composition comprising an arginine deiminase bonded to polyethylene glycol effective to inhibit viral replication.

[0098] In some embodiments the present invention provides methods of inhibiting viral replication in an individual who has been identified as having been infected with a viral infection. The methods comprise administering to the individual an amount of a composition comprising an arginine deiminase bonded to polyethylene glycol effective to inhibit viral replication.

[0099] In some embodiments the composition comprising an arginine deiminase bonded to polyethylene glycol is effective at a concentration of less than 0.1 mM to inhibit viral replication by at least 50% in greater than 50% of cells in an *in vitro* assay to measure viral replication. In some embodiments the composition comprising an arginine deiminase bonded to polyethylene glycol is effective at a concentration of less than 0.05 mM to inhibit viral replication by at least 50% in greater than 50% of cells in an *in vitro* assay to measure viral replication. In some embodiments the composition comprising an arginine deiminase bonded to polyethylene glycol is effective at a concentration of less than 0.01 mM to inhibit viral replication by at least 50% in greater than 50% of cells in an *in vitro* assay to measure viral replication.

[00100] In some embodiments the present invention provides methods of concurrently treating a tumor and inhibiting replication of one or more viruses in an individual. The method comprises administering a therapeutically or prophylactically effective amount of an arginine deiminase covalently bonded via a linking group to polyethylene glycol to the individual. In some embodiments the tumor is selected from the group consisting of melanoma, sarcoma, and hepatoma. In some embodiments the tumor is hepatoma and the virus is HCV. In some embodiments, the presence and/or identity of the tumor is unknown at the time of treatment. In some embodiments the presence and/or identity of the virus is unknown at the time of treatment. In some embodiments the methods further comprise administering a therapeutically effective amount of an additional anti-viral agent prior to, simultaneously with, or following administration of the arginine deiminase.

[00101] In some embodiments the present invention provides methods for modulating nitric oxide levels in an individual comprising administering a therapeutically or prophylactically effective amount of an arginine deiminase bonded to polyethylene glycol to said individual. In some embodiments, modulation is inhibition of nitric oxide levels. In some embodiments the methods further comprise administering a therapeutically or prophylactically effective amount of an additional anti-viral agent prior to, simultaneously with, or following administration of the arginine deiminase. In some embodiments the individual has been identified as having been infected with one or more viruses.

[00102] In some embodiments the present invention provides methods to determine the sensitivity of viral replication to modulating levels of arginine contacting a sample with a composition comprising arginine deiminase bonded to polyethylene glycol and measuring levels of viral RNA or products of viral RNA. Methods of measuring levels of viral RNA or products thereof are well known to those of ordinary skill in the art.

[00103] In some embodiments the present invention provides methods of selectively inhibiting viral replication in an individual infected with one or more viruses. The methods comprise administering a therapeutically or prophylactically effective amount of a composition comprising an arginine deiminase bonded to polyethylene glycol to the individual. In some embodiments the virus is HCV. In some embodiments the SI is above 10, above 15, above 20, or above 25.

[00104] In some embodiments the present invention provides methods for improving liver function in an individual comprising administering a therapeutically or prophylactically effective amount of a composition comprising arginine deiminase bonded to polyethylene glycol to said individual.

[00105] Those of skill in the art are readily able to determine the quality of liver function. In some embodiments, the relative quantity of one or more markers is compared between a healthy patient and a patient with a liver disease or disorder.

[00106] In some embodiments, liver function is assessed using the Child-Pugh scale or the Mayo End-stage Liver Disease (MELD) score. The Child-Pugh scale of grading liver function uses several factors to predict mortality in liver disease. Factors considered in the Child Pugh scale include billrubin levels, creatine levels, international normalized ratio (INR; also known as prothrombin time (measure of blood's ability to clot)), presence of ascites in the abdomen, and grade of encephalopathy. Grades are assigned to levels of increasing abnormality of liver

function; the grade "A" reflects a Child-Pugh score of 5-6 points and indicates the lowest level of liver abnormality. The grade "B" reflects a Child-Pugh score of 7-9 points and indicates an intermediate level of liver abnormality. The grade "C" reflects a Child-Pugh score of 10-15 points and indicates the highest level of liver abnormality. The MELD scale of grading liver function considers billrubin levels, creatine levels, and international normalized ratio.

[00107] In some embodiments the liver function of the individual prior to administration of the arginine deiminase bonded to polyethylene glycol is Child-Pugh level A, level B, or level C.

[00108] In some embodiments the present invention provides methods for identifying an individual identified as having one or more viral infections as susceptible to arginine deprivation therapy. The methods comprise obtaining a viral sample from the individual and comparing viral replication in the sample in the presence and absence of a composition comprising arginine deiminase bonded to polyethylene glycol under conditions suitable for viral replication. In some embodiments an inhibition of viral replication of at least 40%, at least 50%, or at least 80% in the sample contacted with ADI-PEG is indicative of an individual who is a candidate for arginine deprivation therapy and an inhibition of viral replication by ADI-PEG of less than 40%, less than 30%, or less than 20% is indicative of an individual who is not a candidate for arginine deprivation therapy.

[00109] In some embodiments, the present invention provides methods for treating one or more viral infections in an individual. The methods comprise determining if the individual is a candidate for arginine deprivation therapy as described above and treating the individual with arginine deprivation therapy if the individual is a candidate for arginine deprivation therapy and treating the individual with conventional antiviral treatment if the individual is not a candidate for arginine deprivation therapy.

[00110] Methods of determining the most effective means and dosage of administration are well known to those of skill in the art. In some embodiments twice weekly dosing over a period of at least several weeks is used. Often the anti-viral compounds will be administered for extended periods of time and may be administered for the lifetime of the individual. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art. Single or multiple administrations can be carried out with one dose level and pattern being selected by the administrator.

[00111] The dosage administered will, of course, vary depending upon known factors, such as the pharmacodynamic characteristics of the particular agent and its mode and route of

administration; the age, health and/or weight of the individual; the nature and extent of the symptoms; the kind of concurrent treatment; the frequency of treatment; the symptoms exhibited by the individual, and the effect desired.

[00112] Symptoms or criteria for response to anti-viral treatment center around the level of viral replication in the case of most viral infections. Tests for viral circulating viral RNA levels and changes therein are standard and can be applied in cells and animals, including humans. In human patients, tests for liver activities may be performed. One exemplary test is the ALT (Serum Glutamic Pyruvic Transaminase) test. ALT is an enzyme found primarily in the liver but also to a lesser degree, the heart and other tissues, and is useful in diagnosing liver function. The normal adult range for humans is from 0 to about 48 U/L with an optimal adult reading of about 24 U/L. Improvement in one or more of these criteria signals an effective dosage or treatment.

[00113] The compounds may be administered in admixture with suitable pharmaceutical diluents, extenders, excipients, or carriers (collectively referred to herein as a pharmaceutically acceptable carrier) selected with respect to the intended form of administration and as consistent with conventional pharmaceutical practices. For example, in some embodiments ADI-PEG may be mixed with a phosphate buffered saline solution, or any other appropriate solution known to those skilled in the art, prior to injection. The ADI-PEG formulation may be administered as a solid (lyophilate) or as a liquid formulation, as desired.

[00114] The compositions of the present invention are formulated according to the mode of administration to be used. In cases where pharmaceutical compositions are injectable pharmaceutical compositions, they are sterile, pyrogen free and particulate free. In some embodiments the compositions are isotonic formulations. In some embodiments additives for isotonicity can include one or more of sodium chloride, dextrose, mannitol, sorbitol and lactose. In some embodiments, the compositions are provided as isotonic solutions such as phosphate buffered saline. Stabilizers for the compositions include gelatin and albumin in some embodiments.

[00115] The present invention also provides methods of treating a broad spectrum of genetically diverse viruses in a patient comprising administering to the individual a therapeutically effective amount of a compound comprising ADI covalently bonded via a linking group to polyethylene glycol.

[00116] Combination Therapy

[00117] The compounds of the present invention may additionally be combined with other antiviral compounds to provide a combination therapy. Any known anti-viral may be combined with the compositions of the present invention, as long as the combination does not eliminate the antiviral activity of the compound of ADI-PEG. In the case of HIV a combination therapy of ADI-PEG with AZT, TC-3 or protease inhibitors may be more effective than either agent individually. In the case of hepatitis, a combination of ADI-PEG with one or more of cyclovir, famciclovir or valacyclovir, ribavirin, interferon or beta globulin is administered as a combination therapy. For herpes, a recombinant alpha interferon can be used as a combination therapy with ADI-PEG.

[00118] Other anti-viral agents suitable for use in combination therapy are known to the art-skilled and include, without limitation, one or more of AZT (zidovudine, Retrovir), ddI (didanosine, Videx), 3TC (lamivudine, Epivir), d4T (stavudine, Zerit), abacavir (Ziagen) , ddC (zalcitabine, Hivid), nevirapine (Viramune), Delavirdine (Rescriptor), indinavir (Crixivan), ritonavir (Norvir), nelfinavir (Viracept), saquinavir, lopinavir/ ritonavir (Kaletra), Amprenavir (Agenerase) Atazanavir, tipranavir, fusion inhibitor T-20, Interleukin-2, hydroxyurea, AR177 (Zintevir), fomivirsen sodium (Vitravene), GEM 132, GEM 91, GEM 92, AMD 3100, n-docosanol (1-docosanol), PRO2000, T-1249, T-20, arbidol, SP-303 (Virend), hypericin (VIMRxyn), MDL 28574, SC-48334, ADA, imiquimod (Aldera), ISIS 5320, resiquimod, adefovir dipivoxil (Preveon), DAPD, emtricitabine (Coviracil), entecavir, lamivudine (Zeffix, Epivir-HBV, Heptovir, Heptodin), amantadine (Symmetrel), oseltamivir (Tamiflu), pirodavir, pleconaril (VP-63843), ribavirin (Virazid/Virazide/Virazole), rimantadine (Flumadine), WIN 54954, zanamivir (Relenza), foscarnet (Foscavir), maribavir, ABT-378, atevirdine mesylate, calanolide A, capravirine, efavirenz (Sustiva), emivirine (Coactinon), GW420 867X (aka HBY 1293), HBY 097, L-697,66I, loviride, MIV-150, PETT-5, R165335-TMC125, talviraline, tivirapine, trovirdine, acyclovir (Zovirax), brivudin (Helpin, Zostrex), cidofovir (Vistide (i.v.); Forvade (topical)), cyclic HPMPC, famciclovir (Famvir), fiacitabine, fialuridine, ganciclovir (Cymvene/Cytovene), GW-273175X, idoxuridine (Herpid, Kerecid/Herplex Liquifilm, Idoxene, Virudox, Iduridin, Stoxil), lobucavir, netivudine (Zonavir), penciclovir (Vectavir/Denavir), sorivudine (Usevir), trifluridine (Viroptic), valaciclovir (Valtrex; Zelitrex), valomaciclovir stearate (MIV-606), vidarabine (Vira-A), 935U83, abacavir (Ziagen/Trizivir), adefovir, adefovir dipivoxil (Preveon), alovudine, AzdU, CS-92, DAPD, didanosine (Videx), dOTC, emtricitabine (Coviracil), fozivudine tidoxil, lamivudine (Epivir/Combivir/Trizivir), lobucavir, lodenosine,

stavudine (Zerit), tenofovir (Viread), tenofovir disoproxil fumarate, zalcitabine (Hivid), zidovudine (Retrovir), A-77003, AG7088, amprenavir (Agenerase), BMS-232632, delavirdine (Rescriptor), DMP-323, DMP-450, GW 433 908, indinavir (Crixivan), KNI-272, lasinavir, lopinavir (Kaletra), Mozenavir, nelfinavir (Viracept), PD178390, ritonavir (Norvir), RPI 312, saquinavir (Invirase/Fortovase), SC-52151, SDZ PRI 053, tipranavir, U-103017, U-96988, Hydroxyurea (Hydrea), AGI549, foscarnet (Foscavir), LiGLA, Aciclovir - Valaciclovir, Famciclovir, Idoxuridine, Ganciclovir, Foscarnet, Cidofovir, and Adefovir, enfuvirtide, Valcyte, clevudine, thymalfasin, IL-12, among others.

[00119] Combination therapy can be sequential, that is the treatment with one agent first and then the second agent, or it can be treatment with both agents at the same time. The sequential therapy can be within a reasonable time after the completion of the first therapy before beginning the second therapy. The treatment with both agents at the same time can be in the same daily dose or in separate doses. For example in some embodiments treatment with one agent occurs on day 1 and with the other on day 2. The exact regimen will depend on the disease being treated, the severity of the infection and the response to the treatment.

[00120] The *in vivo* means of administration of the compounds of the present invention will vary depending upon the intended application. As one skilled in the art will recognize, administration of the ADI-PEG composition of the present invention can be carried out, for example, by inhalation or suppository or to mucosal tissue such as by lavage to vaginal, rectal, urethral, buccal and sublingual tissue, orally, topically, intranasally, intraperitoneally, parenterally, intravenously, intralymphatically, intratumorly, intramuscularly, interstitially, intra-arterially, subcutaneously, intraoccularly, intrasynovial, transepithelial, and transdermally. The compounds of the present invention can be administered in oral dosage forms as tablets, capsules, pills, powders, granules, elixirs, tinctures, suspensions, syrups, and emulsions. The compounds may also be administered in intravenous (bolus or infusion), intraperitoneal, subcutaneous, or intramuscular form, all using dosage forms well known to those of ordinary skill in the pharmaceutical arts.

[00121] EXAMPLES

[00122] The invention is further demonstrated in the following examples, which are for purposes of illustration, and are not intended to limit the scope of the present invention.

[00123] Example 1: Production of Recombinant ADI

[00124] Cultures of *Mycoplasma arginini* (ATCC 23243), *Mycoplasma hominus* (ATCC 23114) and *Mycoplasma arthritides* (ATCC 23192) were obtained from the American Type Culture Collection, Rockville, Maryland.

[00125] Arginine deiminase was cloned from *Mycoplasma arginini*, *Mycoplasma hominus* and *Mycoplasma arthritides* and expressed in *E. coli* as previously described by S. Misawa et al, J. Biotechnology, 36:145-155 (1994), the disclosure of which is hereby incorporated herein by reference in its entirety. Characterization, by methods known to those skilled in the art, of each of the proteins with respect to specific enzyme activity, K_m, V_{max} and pH optima revealed that they were biochemically indistinguishable from each other. The pH optima was determined using a citrate buffer (pH 5-6.5), a phosphate buffer (pH 6.5-7.5) and a borate buffer (pH 7.5-8.5). The K_m and V_{max} were determined by incubating the enzyme with various concentrations of arginine and quantifying citrulline production. The K_m for the various enzymes was about 0.02 to 0.06 μM and the V_{max} was about 15-20 μmol/min/mg, the values of which are within standard error of each other.

[00126] The arginine deiminase genes were amplified by polymerase chain reaction using the following primer pair derived from the published sequence of *M. arginini*, as described, for example, by T. Ohno et al, Infect. Immun., 58:3788-3795 (1990), the disclosure of which is hereby incorporated by reference herein in its entirety:

5'-GCAATCGATGTGTATTTGACAGT-3' (SEQ ID NO:11)

5'-TGAGGATCCTTACTACCACTTAACATCTTTACG-3' (SEQ ID NO:12)

[00127] The polymerase chain reaction product was cloned as a Bam H1-Hind III fragment into expression plasmid pQE16. DNA sequence analysis indicated this fragment had the same sequence for the arginine deiminase gene as described by Ohno et al, Infect. Immun., supra. The five TGA codons in the ADI gene which encode tryptophan in Mycoplasma were changed to TGG codons by oligonucleotide-directed mutagenesis prior to gene expression in E. coli, as taught, for example, by J.R. Sayers et al, Biotechniques, 13:592-596 (1992). Recombinant ADI was expressed in inclusion bodies at levels of 10% of total cell protein.

[00128] The proteins from each of the above three species of Mycoplasma have approximately 95% homology and are readily purified by column chromatography. Approximately 1.2 g of pure protein may be isolated from 1 liter of fermentation broth. Recombinant ADI is stable for about 2 weeks at 37°C and for at least 8 months when stored at

4°C. As determined by methods known to those skilled in the art, the proteins had a high affinity for arginine (0.04 μM), and a physiological pH optima of about 7.2 to about 7.4.

[00129] Example 2: Renaturation and Purification of Recombinant ADI

[00130] ADI protein was renatured, with minor modifications, as described by Misawa et al, J. Biotechnology, 36:145-155 (1994), the disclosure of which is hereby incorporated herein by reference in its entirety. 100 g of cell paste was resuspended in 800 ml of 10 mM K2PO4 pH 7.0, 1 mM EDTA (buffer 1) and the cells were disrupted by two passes in a Microfluidizer (Microfluidics Corporation, Newton, MA). Triton X-100 was added to achieve a final concentration of 4% (v/v). The homogenate was stirred for 30 min at 4°C, then centrifuged for 30 min at 13,000 g. The pellet was collected and resuspended in one liter of buffer 1 containing 0.5% Triton X-100. The solution was diafiltered against 5 volumes of denaturation buffer (50 mM Tris HCl, pH 8.5, 10 mM DTT) using hollow-fiber cartridges with 100 kD retention rating (Microgon Inc., Laguna Hills, CA). Guanidine HCl was added to achieve a final concentration of 6 M and the solution was stirred for 15 min at 4°C. The solution was diluted 100-fold into refolding buffer 1, 10 mm K_2PO_4, pH 7.0 and stirred for 48 hours at 15°C, particulates were removed by centrifugation at 15,000 x g.

[00131] The resulting supernatant was concentrated on a Q Sepharose Past Flow (Pharmacia Inc., Piscataway, NJ) column preequilabrated in refolding buffer. ADI was eluted using refolding buffer containing 0.2 M NaCl. The purification procedure yielded ADI protein, which was >95% pure as estimated by SDS-PAGE analysis. Eight g of pure renatured ADI protein was produced from 1 kg of cell paste which corresponds to 200 mg purified ADI per liter of fermentation.

[00132] ADI activity was determined by micro-modification of the method described by Oginsky et al, Meth. Enzymol., (1957) 3:639-642. Ten μl samples in 0.1 M Na_2PO_4, pH 7.0 (BUN assay buffer) were placed in a 96 well microliter plate, 40 μl of 0.5 mM arginine in BUN assay buffer was added, and the plate was covered and incubated at 37°C for 15 minutes. Twenty μl of complete BUN reagent (Sigma Diagnostics) was added and the plate was incubated for 10 minutes at 100°C. The plate was then cooled to 22°C and analyzed at 490 nm by a microliter plate reader (Molecular Devices, Inc). One IU is the amount of enzyme which converts 1 μmole of L-arginine to L-citrulline per minute. Protein concentrations were determined using Pierce Coomassie Blue Protein Assay Reagent (Pierce Co., Rockford, IL) with bovine serum albumin as a standard. The enzyme activity of the purified ADI preparations was

17-25 IU/mg.

[00133] Example 3: Attachment of PEG to ADI

[00134] PEG was covalently bonded to ADI in a 100 mM phosphate buffer, pH 7.4. Briefly, ADI in phosphate buffer was mixed with a 100 molar excess of PEG. The reaction was stirred at room temperature for 1 hour, then the mixture was extensively dialysed to remove unincorporated PEG.

[00135] A first experiment was performed where the effect of the linking group used in the PEG-ADI compositions was evaluated. PEG10,000 and ADI were covalently bonded via four different linking groups: an ester group or maleimide group, including SS, SSA, SPA and SSPA, where each PEG molecule had an average molecular weight of 5,000, 10,000, 12,000, 20,000, 30,000 and 40,000; an epoxy group, PEG-epoxy, where each PEG molecule had an average molecular weight of 5,000; and a branched PEG group, PEG2-NHS, where each PEG molecule had an average molecular weight of 10,000, 20,000 and 40,000.

[00136] Five IU of the resulting compositions were injected into mice (5 mice in each group). To determine the serum levels of arginine, the mice were bled from the retro orbital plexus (100 µl). Immediately following collection an equal volume of 50% (w/v) of trichloroacetic acid was added. The precipitate was removed by centrifugation (13,000 x g for 30 minutes) and the supernatant removed and stored frozen at –70°C. The samples were then analyzed using an automated amino acid analyzer and reagents from Beckman Instruments using protocols supplied by the manufacturer. The limits of sensitivity for citrulline by this method was approximately 2-6 µM and the reproducibility of measurements within about 8%. The amount of serum arginine was determined by amino acid analysis. The linking group covalently bonding the PEG and ADI did not have an appreciable effect on the ability of ADI to reduce serum arginine *in vivo*.

[00137] A second experiment was performed wherein the effect of the linking group and molecular weight of PEG on serum citrulline levels *in vivo* was evaluated. Mice (5 in each group) were given various compositions of ADI and PEG-ADI in an amount of 5.0 IU. To determine the serum levels of citrulline, the mice were bled from the retro orbital plexus (100 µl). Immediately following collection an equal volume of 50% (w/v) of trichloroacetic acid was added. The precipitate was removed by centrifugation (13,000 x g for 30 minutes) and the supernatant removed and stored frozen at -70°C. The samples were then analyzed using an

automated amino acid analyzer and reagents from Beckman Instruments using protocols supplied by the manufacturer. The limits of sensitivity for citrulline by this method was approximately 2-6 μM and the reproducibility of measurements within about 8%. The amount of citrulline was determined, and the area under the curve approximated and expressed as μmol days.

[00138] The results demonstrate that the molecular weight of the PEG determines the effectiveness of the PEG-ADI composition. The effectiveness of the PEG-ADI compositions does not appear to be based on the method or means of attachment of the PEG to ADI.

[00139] The results demonstrate that the optimal molecular weight of PEG is about 20,000. Although PEG30,000 appears to be superior to PEG20,000 in terms of its pharmacodynamics, PEG30,000 is less soluble, which makes it more difficult to work with. The yields, which were based on the recovery of enzyme activity, were about 90% for PEG5,000 and PEG12,000; about 85% for PEG20,000 and about 40% for PEG30,000. Therefore, in some embodiments PEG20,000 appears to be a good compromise between yield and circulating half life, as determined by citrulline production.

[00140] In a third experiment, the dose response of serum arginine depletion and the production of citrulline with ADI-SS-PEG5,000 and ADI-SS-PEG20,000 was determined. Mice (5 in each group) were given a single injection of 0.05 IU, 0.5 IU or 5.0 IU of either ADI-SS-PEG5,000 or ADI-SS-PEG20,000. At indicated times, serum was collected, as described above, and an amino acid analysis was performed to quantify serum arginine and serum citrulline. Both formulations induced a dose dependent decrease in serum arginine and an increase in serum citrulline. However, the effects induced by ADI-SS-PEG20,000 were more pronounced and of longer duration than the effects induced by ADI-SS-PEG5,000.

[00141] Example 4: Circulating Half-Life

[00142] Balb C mice (5 in each group) were injected intravenously with a single 5.0 IU does of either native arginine deiminase or various formulations of arginine deiminase modified with polyethylene glycol. To determine the serum levels of arginine and citrulline, the mice were bled from the retro orbital plexus (100 μl). Immediately following collection an equal volume of 50% (w/v) of trichloro-acetic acid was added. The precipitate was removed by centrifugation (13,000 x g for 30 minutes) and the supernatant removed and stored frozen at -70°C. The samples were then analyzed using an automated amino acid analyzer and reagents from Beckman Instruments using protocols supplied by the manufacturer. The limits of sensitivity

for arginine by this method was approximately 6 pM and the reproducibility of measurements within about 8%.

[00143] A dose dependent decrease in serum arginine levels and a rise in serum citrulline were detected from the single dose administration of native ADI or ADI-SS-PEG. However, the decrease in serum arginine and rise in serum citrulline was short lived, and soon returned to normal. The half-life of arginine depletion is summarized in Table 2 below.

Table 2: Half-Life of Serum Arginine Depletion

Compound	Half-Life in Days
Native ADI	1
ADI-SS-PEG5,000	5
ADI-SS-PEG12,000	15
ADI-SS-PEG20,000	20
ADI-SS-PEG30,000	22

[00144] **Example 5: Antigenicity of PEG modified ADI**

[00145] To determine the antigenicity of native ADI, ADI-SS-PEG5,000, and ADI-SS-PEG20,000, the procedures described in, for example, Park, Anticancer Res., *supra,* and Kamisaki, J. Pharmacol. Exp. Ther., *supra*, were followed.. Briefly, Balb C mice (5 in each group) were intravenously injected weekly for 12 weeks with approximately 0.5 IU (100 μg of protein) of native ADI, ADI-SS-PEG5,000 or ADI-SS-PEG20,000. The animals were bled (0.05 ml) from the retro orbital plexus at the beginning of the experiment and at weeks 4, 8 and 12. The serum was isolated and stored at -70°C. The titers of anti-ADI IgG were determined by ELISA. Fifty μg of ADI was added to each well of a 96 well micro-titer plate and was incubated at room temperature for 4 hours. The plates were rinsed with PBS and then coated with bovine serum albumin (1 mg/ml) to block nonspecific protein binding sites, and stored over night at 4°C. The next day serum from the mice was diluted and added to the wells. After 1 hour the plates were rinsed with PBS and rabbit anti-mouse IgG coupled to peroxidase was added to the

wells. The plates were incubated for 30 min and then the resulting UV absorbance was measured using a micro-titer plate reader. The titer was defined as the highest dilution of the serum which resulted in a two-fold increase from background absorbance (approximately 0.50 OD).

[00146] ADI-SS-PEG5,000 and ADI-SS-PEG20,000 are significantly less antigenic than native ADI. For example, as few as 4 injections of native ADI resulted in a titer of about 10^6, while 4 injections of any of the PEG-ADI formulations failed to produce any measurable antibody. However, after 8 injections, the ADI-PEG5,000 had a titer of about 10^2, while ADI-PEG20,000 did not induce this much of an immune response until after 12 injections. The results demonstrate that attaching PEG to ADI blunts the immune response to the protein.

[00147] Example 6: Application to Humans

[00148] PEG5,000-ADI and PEG20,000-ADI were incubated *ex vivo* with normal human serum and the effects on arginine concentration was determined by amino acid analysis, where the enzyme was found to be fully active and capable of degrading all the detectable arginine with the same kinetics as in the experiments involving mice. The reaction was conducted at a volume of 0.1 ml in a time of 1 hour at 37°C.

[00149] Additionally, the levels of arginine and citrulline in human serum are identical with that found in mice. PEG-proteins circulate longer in humans than they do in mice. For example, the circulating half life of PEG conjugated adenosine deiminase, asparaginase, glucocerbrocidase, uricase, hemoglobulin and superoxide dismutase all have a circulating half life that is 5 to 10 times longer than the same formulations in mice. What this has meant in the past is that the human dose is most often 1/5 to 1/10 of that used in mice. Accordingly, PEG-ADI should circulate even longer in humans than it does in mice.

[00150] Example 7

[00151] The antiviral activity of ADI-PEG20 was tested in a stably HCV RNA replicating cell line AVA5 derived by transfection of a human hepatoblastome cell line Huh7 (Blight et al., Efficient Initiation of HCV RNA Replication in Cell Culture, *Science* 2000 290: 1972-1974).

[00152] ***In vitro* Replication Assay**

[00153] A stable HCV RNA replicating cell line AVA5 derived by transfection of a human

hepatoblastoma cell line Huh7 was used. Dividing cultures of AVA5 cells were treated once daily for three days (media was changed with each addition of compound) with 4 concentrations of test compound (3 cultures per concentration). A total of 6 untreated control cultures, and triplicate cultures treated with 10, 3, and 1 IU/ml α-interferon (active antiviral with no cytotoxicity), and 100, 10 and 1 uM ribavirin (no antiviral activity and cytotoxic) served as controls. HCV RNA and cellular β-actin RNA levels were assessed 24 hours after the last dose of compound using dot blot hybridization. β-actin RNA levels were used to normalize the amount of cellular RNA in each sample. Toxicity analyses were performed on separate plates from those used for the antiviral assays. Cells for the toxicity analyses were cultured and treated with test compounds with the same schedule and under identical culture conditions as used for the antiviral evaluations. Each compound was tested at 4 concentrations, each in triplicate cultures. Uptake of neutral red dye was used to determine the relative level of toxicity 24 hours following the last treatment. The absorbance of internalized dye at 510nm (A_{510}) was used for the quantitative analysis. Values in test cultures were compared to 9 cultures of untreated cells maintained on the same plate as the test cultures. The 50% and 90% effective antiviral concentrations (EC_{50},EC_{90}) and the 50% cytotoxic concentrations (CC_{50}) were calculated and used to generate Selectivity Indexes (CC_{50}/EC_{50}). An S.I. of 10 or greater is considered to be a selective antiviral effect.

[00154] Antiviral activity of ADI-PEG20

[00155] A single dose of ADI-PEG20 (0.01 IU/ml) was added to dividing cultures of these cells when they are at 50% confluence. As a control alpha interferon (10 IU/ml) and ribavirin (100 μM) were used as positive controls. After 3 days of treatment RNA was isolated from the cultures using standard laboratory techniques and assayed using dot blots. The amount of HCV mRNA was determined and compared to the mRNA for actin (which is used as a control). The amount of drug (ADI, alpha interferon or raboviron) required to inhibit 50% of the control levels of HCV mRNA is determined. Any dose of drug that causes a 50% inhibition of actin mRNA is considered to have nonspecific inhibitory activity. The results obtained from this experiment are shown below.

Drug	*% inhibition of HCV mRNA*	*% inhibition of actin*
ADI-PEG20	86%	12%
alpha interferon	92%	11%

ribavirin	25%	98%

These data demonstrate that ADI-PEG inhibits HCV viral replication *in vitro* nearly as well as alpha interferon and much greater than ribavirin.

[00156] Example 8

[00157] Dividing cultures of AVA5 cells were treated with various concentrations of PEG-ADI (or in control experiments alpha interferon or ribavirin) for 3 days. HCV mRNA levels were assayed as above. Cell viability was determined using neutral red. The concentrations which inhibit 50% (IC_{50}) and 90% (IC_{90}) of HCV mRNA levels were determined. The concentration of drug which kills 50% of the cells (CC_{50}) was also determined. The CC_{50}/EC_{50} is calculated to determine the selectivity index (SI). An SI>10 is considered to be a selective inhibition of the viral replication. The results are shown below.

Drug	*CC_{50}*	*IC_{50}*	*IC_{90}*	*SI*
ADI-PEG20	0.335 IU/ml	0.27 IU/ml	0.188 IU/ml	12
alpha interferon	>10000 IU/ml	2.1 IU/ml	9.0 IU/ml	>4762
ribavirin	74μM	>10μM	>10μM	NA

These data confirm that ADI-PEG20 inhibits HCV replication and that this drug is selective.

[00158] Example 9: Antiviral Activity and NO Synthesis in Tumor Patients

[00159] ADI-PEG 20 was tested for anti-tumor activity in patients with hepatocellular cancer also chronically infected with HCV. Viral titers of HCV in the plasma of these patients using standard clinical assays developed by Hoffman La Roche were also determined. Plasma was obtained prior to treatment with ADI-PEG 20. The patients were injected with 160 IU/m^2 of ADI-PEG 20 once a week for 3 weeks. One week following the third injection with ADI-PEG 20, plasma was isolated from the patients and again assayed for HCV titer using the same assay. The results from this experiment are shown below.

Patient Number	*HCV titer Pretreatment*	*HCV titer Post treatment*
1	614,836	485,900
2	1,255,542	254,729
3	328,134	97,535

4	1,466,460	63,902
5	1,187,730	485,190

[00160] These data demonstrate that ADI-PEG treatment of humans chronically infected with HCV results in significantly lower titers of HCV in their plasma. Moreover as alpha interferon is only effective in ~50% of these patients and it frequently requires 3-6 months of treatment to achieved a 50% reduction in HCV titers, it appears that ADI-PEG 20 is much more effective in this regard.

[00161] ADI-SS PEG 20,000 mw was tested in a Phase 2 study of individuals with inoperable HCC according to Richard Simon statistical design for rapid optimal two-stage Phase 2 testing (Simon R. 1989. Optimal two-stage designs for phase II clinical trials. Control Clin Trials 10:1-10; Simon RM, Steinberg SM, Hamilton M, Hildesheim A, Khleif S, Kwak LW, Mackall CL, Schlom J, Topalian SL, Berzofsky JA. 2001. Clinical trial designs for the early clinical development of therapeutic cancer vaccines. J Clin Oncol 19:1848-1854.). This testing was performed under approval by the Italian Health Ministry at the Pascale National Cancer Institute in Naples, Italy and with the approval of the local institutional review board. All subjects were provided informed consent according to the Declaration of Helsinki. A total of 18 individuals with inoperable HCC were enrolled in this study who were chronically infected with HCV (Izzo submitted). During this study 3 died from progressive disease and failed to receive all 3 cycles of treatment and thus were excluded form further analysis. All remaining 15 subjects received 3 cycles (each consisting of 4 once a week injections) of ADI-SS PEG 20,000 mw at the Optimum Biological Dose. The Optimum Biological Dose was defined as that amount of ADI-SS PEG 20,000 mw which lowered plasma arginine from a resting level of ~130 μM to below the level of detection (<2 μM) for at least 7 days (~160 IU/m^2).

[00162] The action this therapy had on the tumors was assessed by CT scans once every 4 weeks. Response was defined as either Progressive disease (PD), stable disease (SD), partial response (PR) or complete response (CR) according to standard National Cancer Institute (NCI) criteria. The results from this testing indicated that in the 15 subjects with HCC and HCV the following responses were seen:

Status of Disease	Number of Subjects
Complete Response (CR)	2
Partial Response (PR)	7
Stable Disease (SD)	10

[00163] None of the subjects had received any systemic anti-tumor treatment (or anti-viral treatment) either prior to or during this study. Clinical laboratory testing was performed at least twice a week during the study and plasma samples were collected once a week and archived frozen at -70°C. It was these frozen archived plasma samples that were later tested for HCV.

[00164] Assay for HCV titers and serotyping of human plasma samples

[00165] HCV viral titers were determined in the hospital infectious disease clinical laboratory using a standard clinical polymerase-chain-reaction (PCR) assay, Cobas Amplicor HCV Monitor Test, version 2.0; Roche Diagnostics (Germer 1999). The genotype was similarly determined. Viral titers were determined on plasma samples collected prior to ADI-SS PEG 20,000 mw treatment and after 12 weeks of therapy.

[00166] NO synthesis

[00167] Treatment with ADI-SS PEG 20,000 mw results in a dose dependent decrease in plasma arginine and concomitant decrease in NO synthesis (data not shown). Although this treatment significantly decreased NO levels, there was no measurable effect of this treatment on blood pressure or heart rate.

[00168] The following Table 3 lists the effect of ADI-SS PEG 20,000 mw on Hepatitis C Titers and Liver Function Tests.

[00169] **Table 3: Effect of ADI-SS PEG 20,000 mw on Hepatitis C Titers and Liver Function Tests.**

Patient Number	Response	HCV Titer pre Rx	HCV Titer post Rx	Titer % Decrease	Sero Type	ALT Pre Rx	ALT Post Rx	AST Pre Rx	AST Post Rx	Bilirubin Total Pre Rx	Bilirubin Total Post Rx
1	PR	614,836	<6000	> 99	1b	271	227	245	182	1.16	0.92
2	SD	1,466,400	63,902	96	1b	104	115	101	98	0.69	0.33
3	SD	269,000	28,200	90	1b	118	81	76	71	1.32	0.60
4	SD	1,187,730	40,200	97	1b	153	110	145	80	3.4	0.45
5	PR	614836	40,200	93	1b	87	122	85	108	1.8	0.45
6	CR	328,134	173,000	47	1b	63	66	51	47	0.94	0.45
7	CR	<6000	<6000	-	1b	57	67	65	74	1.72	0.45
8	SD	676,000	120,000	82	1b	57	67	65	74	1.72	0.45
9	SD	<6000	<6000	-	1b	63	25	37	14	1.73	0.60
10	PR	1,950,000	921,000	53	1b	68	58	69	47	1.70	0.73
11	SD	386,000	331,000	14	1b	89	153	95	151	2.83	3.1
12	PR	2,830,000	3,390,000	increase	1b	77	65	85	83	0.72	0.83
13	SD	689,000	1,010,000	increase	2c	66	54	68	47	1.66	0.99
14	SD	351,000	690,000	increase	2c	115	137	111	152	3.13	3.1
15	SD	801,000	1,210,000	increase	2c	79	87	85	76	1.68	1.80

[00170] Note All post RX values are after 3 cycles at the OBD.

[00171] Each of the patents, Genbank accession numbers, patent applications and publications described herein are hereby incorporated by reference herein in their entirety.

[00172] Various modifications of the invention, in addition to those described herein, will be apparent to one skilled in the art in view of the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

What is claimed is:

1. A method of inhibiting replication of one or more viruses in an individual comprising administering to said individual a composition comprising an arginine deiminase bonded to polyethylene glycol in an amount effective to inhibit viral replication in said individual.

2. The method of claim 1 further comprising the step of administering to said individual one or more compounds selected from the group consisting of antibiotics, anti-virals, antifungals, and anti-protozoan drugs.

3. The method of claim 1 further comprising the step of administering to said individual one or more other anti-viral compounds.

4. The method of claim 2 wherein said anti-viral compounds are one or more of azidovudine (AZT), didanosine (dideoxyinosine, ddI), d4T, zalcitabine (dideoxycytosine, ddC), nevirapine, lamivudine (epivir, 3TC), saquinavir (Invirase), ritonavir (Norvir), indinavir (Crixivan), delavirdine (Rescriptor), pegylated (PEG) interferon-α (IFN), or ribavirin

5. The method of claim 1 wherein said composition is administered intramuscularly, intradermally, or intraperitoneally.

6. The method of claim 1 wherein said composition comprising an arginine deiminase bonded to polyethylene glycol is effective at a concentration of less than about 1 mM to inhibit viral replication by at least 50% in greater than 50% of cells in an assay to measure viral replication.

7. The method of claim 1 wherein the amount of arginine deiminase bonded to polyethylene glycol effective to inhibit viral replication is between about 40 IU/m^2 and about 160 IU/m^2 per week.

8. The method of claim 1 wherein the amount of arginine deiminase bonded to polyethylene glycol effective to inhibit viral replication is about 160 IU/m^2 per week.

9. The method of claim 1 wherein the amount of arginine deiminase bonded to polyethylene glycol effective to inhibit viral replication lowers plasma arginine levels to less than 5 μM.

10. The method of claim 1 wherein the arginine deiminase is covalently bonded via a linking group to polyethylene glycol, wherein each of said polyethylene glycol molecules has a molecular weight of about 10,000 to about 30,000.

11. The method of claim 1 wherein each of said polyethylene glycol molecules has a molecular weight of about 20,000.

12. The method of claim 10 wherein the linking group is selected from the group consisting of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group, and a histidine group, and combinations thereof.

13. The method of claim 10 wherein the linking group is succinimidyl succinate.

14. The method of claim 1 wherein from about 7 to about 15 polyethylene glycol molecules are bonded to arginine deiminase.

15. The method of claim 1 wherein from about 9 to about 12 said polyethylene glycol molecules are bonded to arginine deiminase.

16. The method of claim 1 wherein said arginine deiminase is derived from a microorganism of the genus *Mycoplasma*.

17. The method of claim 16 wherein said microorganism is selected from the group consisting of *Mycoplasma arginini*, *Mycoplasma hominus*, *Mycoplasma arthritides* and combinations thereof.

18. The method of claim 1 wherein the arginine deiminase has an amino acid sequence of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 14, 15, 16, 17, 18, 19, 20 or 21.

19. The method of claim 1 wherein the arginine deiminase has an amino acid sequence of SEQ ID NO: 1or 4.

20. The method of claim 1 wherein the virus is a hepatitis virus.

21. The method of claim 1 wherein the virus is HCV.

22. The method of claim 1 wherein the virus is HCV1b.

23. A method of treating an individual who is suspected of having been exposed to one or more viruses comprising the step of administering to said individual an amount of a composition comprising an arginine deiminase bonded to polyethylene glycol effective to inhibit viral replication in said individual.

24. A method of inhibiting viral replication in an individual at risk for one or more viruses comprising administering to said individual an amount of a composition comprising an arginine deiminase bonded to polyethylene glycol effective to inhibit viral replication in said individual.

25. A method of inhibiting viral replication in an individual who has been identified as having been infected with one or more viruses comprising administering to said individual an amount of a composition comprising an arginine deiminase bonded to polyethylene glycol effective to inhibit viral replication in said individual.

26. A method of concurrently treating a tumor and inhibiting replication of one or more viruses in an individual, said method comprising the step of administering a therapeutically or prophylactically effective amount of a composition comprising arginine deiminase covalently bonded via a linking group to polyethylene glycol to said individual effective to inhibit tumor growth and inhibit viral replication.

27. The method of claim 26 wherein the individual has been identified as having been infected with one or more viral infections prior to administration of the composition.

28. The method of claim 26 wherein the tumor is melanoma, sarcoma, or hepatoma.

29. The method of claim 26 wherein the tumor is hepatocellular carcinoma.

30. The method of any one of claims 23-27 wherein the virus is a hepatitis virus.

31. The method of any one of claims 23-27 wherein said virus is HCV.

32. The method of claim 26 wherein said tumor is hepatocellular carcinoma and said virus is HCV.

33. A method for modulating nitric oxide levels in an individual comprising administering an amount of an arginine deiminase bonded to polyethylene glycol effective to modulate nitric oxide to said individual.

34. The method of claim 33 wherein the arginine deiminase is covalently bonded via a linking group to polyethylene glycol, wherein each of said polyethylene glycol molecules has a molecular weight of about 10,000 to about 30,000.

35. The method of claim 33 wherein each of said polyethylene glycol molecules has a molecular weight of about 20,000.

36. The method of claim 34 wherein the linking group is one or more of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group or a histidine group.

37. The method of claim 34 wherein from about 7 to about 12 polyethylene glycol molecules are bonded to arginine deiminase.

38. The method of claim 33 wherein said arginine deiminase is derived from a microorganism of the genus *Mycoplasma*.

39. A method to determine the sensitivity of viral replication to modulating levels of arginine comprising:

(a) contacting a sample comprising one or more viruses with a composition comprising arginine deiminase bonded to polyethylene glycol; and

(b) comparing levels of viral replication in the presence and absence of the composition comprising arginine deiminase bonded to polyethylene glycol;

wherein decreased viral replication in samples contacted with arginine deiminase is indicative of viral sensitivity to arginine deiminase.

40. A method to determine the sensitivity of viral replication to modulating levels of nitric oxide comprising:

(a) contacting a sample comprising one or more viruses with a composition comprising arginine deiminase bonded to polyethylene glycol; and

(b) comparing levels of viral replication in the presence and absence of the

composition comprising arginine deiminase bonded to polyethylene glycol,
wherein decreased viral replication in samples contacted with arginine deiminase is indicative of viral sensitivity to nitric oxide.

41. The method of any one of claims 2 or 3 wherein said compound is administered to said individual simultaneously with the administration of said composition comprising arginine deiminase bonded to polyethylene glycol.

42. A method of selectively inhibiting viral replication in an individual in need thereof comprising administering a therapeutically or prophylactically effective amount of a composition comprising an arginine deiminase bonded to polyethylene glycol to said individual.

43. The method of claim 42 wherein the virus is HCV.

44. A method for improving liver function in an individual comprising administering a therapeutically effective amount of a composition comprising arginine deiminase bonded to polyethylene glycol to said individual.

45. The method of claim 44 wherein liver function is assessed using the Child-Pugh scale or the Mayo End-stage Liver Disease score.

46. The method of claim 44 wherein liver function is assessed by measuring at least one marker of liver function, wherein the marker is billrubin, albumin, prothrombin time, presence of ascites, or grade of enephalopathy.

47. The method of claim 44 wherein the liver function of said individual prior to administration of the composition comprising arginine deiminase bonded to polyethylene glycol is Child-Pugh level A.

48. The method of claim 44 wherein the liver function of said individual prior to administration of the composition comprising arginine deiminase bonded to polyethylene glycol is Child-Pugh level B.

49. The method of claim 44 wherein the liver function of said individual prior to administration of the composition comprising arginine deiminase bonded to polyethylene glycol is Child-Pugh level C.

50. A method for identifying an individual as susceptible to arginine deprivation therapy, said individual identified as having one or more viral infections, the method comprising:

a) obtaining a sample comprising one or more viruses from the individual; and

b) comparing viral replication in the sample contacted with a composition comprising arginine deiminase bonded to polyethylene glycol under conditions suitable for viral replication to viral replication in the sample in the absence of a composition comprising arginine deiminase bonded to polyethylene glycol, wherein an inhibition of viral replication of at least 40% in said sample is indicative of an individual who is a candidate for arginine deprivation therapy and an inhibition of viral replication of less than 40% is indicative of an individual who is not a candidate for arginine deprivation therapy.

51. A method of treating one or more viruses in an individual comprising:

a) determining if the individual is a candidate for arginine deprivation therapy according to claim 50;

b) treating the individual with arginine deprivation therapy if the individual is a candidate for arginine deprivation therapy; and

c) treating the individual with conventional antiviral treatment if the individual is not a candidate for arginine deprivation therapy.

METHODS FOR INHIBITING VIRAL REPLICATION *IN VIVO*

ABSTRACT OF THE DISCLOSURE

[00173] The present invention is directed to methods of modulating viral replication *in vivo* comprising administering to an individual a therapeutically or prophylactically effective amount of a composition comprising arginine deiminase modified with polyethylene glycol, to methods of concurrently modulating viral replication and treating cancer, and to methods of modulating nitric oxide levels in a patient, among others.

1. A method of inhibiting HCV replication in an individual comprising administering to said individual a composition comprising an arginine deiminase bonded to polyethylene glycol in an amount effective to inhibit HCV replication in said individual.

2. The method of claim 1 further comprising the step of administering to said individual one or more compounds selected from the group consisting of antibiotics, anti-virals, antifungals, and anti-protozoan drugs.

3. The method of claim 1 further comprising the step of administering to said individual one or more conventional antiviral compounds.

4. The method of claim 2 wherein said anti-viral compounds are one or more of
azidovudine (AZT), didanosine (dideoxyinosine, ddI), d4T, zalcitabine (dideoxycytosine, ddC), nevirapine, lamivudine (epivir, 3TC), saquinavir (Invirase), ritonavir (Norvir), indinavir (Crixivan), delavirdine (Rescriptor), pegylated (PEG) interferon-α (IFN), or ribavirin.

5. The method of claim 1 wherein said composition is administered intramuscularly, intradermally, or intraperitoneally.

6. The method of claim 1 wherein said composition comprising an arginine deiminase bonded to polyethylene glycol is effective at a concentration of less than 1 mM to inhibit viral replication by at least 50%.

7. The method of claim 1 wherein the amount of arginine deiminase bonded to
polyethylene glycol effective to inhibit viral replication is between about 40 IU/m^2 and about 160 IU/m^2 per week.

8. The method of claim 1 wherein the amount of arginine deiminase bonded to

polyethylene glycol effective to inhibit viral replication is about 160 IU/m^2 per week.

9. The method of claim 1 wherein the amount of arginine deiminase bonded to
polyethylene glycol effective to inhibit viral replication lowers plasma arginine levels to less
than 5 μM.

10. The method of claim 1 wherein the arginine deiminase is covalently bonded via a linking group to polyethylene glycol, wherein each of said polyethylene glycol molecules has a molecular weight of about 10,000 to about 30,000.

11. The method of claim 1 wherein each of said polyethylene glycol molecules has a molecular weight of about 20,000.

12. The method of claim 10 wherein the linking group is selected from the group
consisting of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group, and a histidine group, and combinations thereof.

13. The method of claim 10 wherein the linking group is succinimidyl succinate.

14. The method of claim 1 wherein from about 7 to about 15 polyethylene glycol
molecules are bonded to arginine deiminase.

15. The method of claim 1 wherein from about 9 to about 12 said polyethylene glycol molecules are bonded to arginine deiminase.

16. The method of claim 1 wherein said arginine deiminase is derived from a microorganism of the genus *Mycoplasma*.

17. The method of claim 16 wherein said microorganism is selected from the group consisting of *Mycoplasma arginini, Mycoplasma hominus, Mycoplasma arthritides* and combinations thereof.

18. The method of claim 1 wherein the arginine deiminase has an amino acid sequence of SEQ ID NO: 1,2,3,4,5,6,7,8,9, 10, 13, 14, 15, 16, 17, 18, 19, 20 or 21.

19. The method of claim 1 wherein the arginine deiminase has an amino acid sequence of SEQ ID NO: 1 or 4.

20 - 21. Cancelled.

22. The method of claim 1 wherein the virus is HCV1b.

23 - 24. Cancelled.

25. A method of inhibiting HCV replication in an individual who has been identified as having been infected with HCV comprising administering to said individual an amount of a composition comprising an arginine deiminase bonded to polyethylene glycol effective to inhibit HCV replication in said individual.

26 - 40. Cancelled.

41. The method of any one of claims 2 or 3 wherein said compound is administered
to said individual simultaneously with the administration of said composition comprising arginine deiminase bonded to polyethylene glycol.

42. A method of selectively inhibiting HCV replication in an individual in need
thereof comprising administering a therapeutically or prophylactically effective amount of a
composition comprising an arginine deiminase bonded to polyethylene glycol to said individual.

43 - 51. Cancelled

52. The method of claim 3 wherein the one or more conventional antiviral medicaments are selected from the group consisting of cyclovir, famciclovir, valacyclovir, ribavirin, interferon or beta globulin.

53. A method of reducing HCV viral titer in an individual comprising administering to said individual a composition comprising an arginine deiminase bonded to polyethylene glycol in an amount effective to reduce HCV viral titer in said individual.

54. The method of claim 53 further comprising the step of administering to said individual one or more conventional antiviral compounds.

55. The method of claim 53 wherein said composition is administered intramuscularly, intradermally, or intraperitoneally.

56. The method of claim 53 wherein said composition comprising an arginine deiminase bonded to polyethylene glycol is effective at a concentration of less than 1 mM to reduce HCV viral titer by at least 50%.

57. The method of claim 53 wherein the amount of arginine deiminase bonded to polyethylene glycol effective to reduce HCV viral titer is between about 40 IU/m^2 and about 160 IU/m^2 per week.

58. The method of claim 53 wherein the amount of arginine deiminase bonded to polyethylene glycol effective to reduce HCV viral titer is about 160 IU/m^2 per week.

59. The method of claim 53 wherein the amount of arginine deiminase bonded to polyethylene glycol effective to reduce HCV viral titer lowers plasma arginine levels to less than 5 μM.

60. The method of claim 53 wherein the arginine deiminase is covalently bonded via a linking group to polyethylene glycol, wherein each of said polyethylene glycol molecules has a molecular weight of about 10,000 to about 30,000.

61. The method of claim 53 wherein each of said polyethylene glycol molecules has a molecular weight of about 20,000.

62. The method of claim 60 wherein the linking group is selected from the group consisting of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group, and a histidine group, and combinations thereof.

63. The method of claim 60 wherein the linking group is succinimidyl succinate.

64. The method of claim 53 wherein from about 7 to about 15 polyethylene glycol molecules are bonded to arginine deiminase.

65. The method of claim 53 wherein from about 9 to about 12 said polyethylene glycol molecules are bonded to arginine deiminase.

66. The method of claim 53 wherein said arginine deiminase is derived from a microorganism of the genus *Mycoplasma*.

67. The method of claim 66 wherein said microorganism is selected from the group consisting of *Mycoplasma arginini, Mycoplasma hominus, Mycoplasma arthritides* and combinations thereof.

68. The method of claim 53 wherein the arginine deiminase has an amino acid sequence of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 14, 15, 16, 17, 18, 19, 20 or 21.

69. The method of claim 53 wherein the arginine deiminase has an amino acid sequence of SEQ ID NO: 1 or 4.

70. The method of claim 53 wherein the virus is HCV1b.

71. The method of claim 54 wherein the one or more conventional antiviral medicaments are selected from the group consisting of cyclovir, famciclovir, valacyclovir, ribavirin, interferon or beta globulin.

72. The method of claim 53 wherein the amount of arginine deiminase bonded to polyethylene glycol administered to the individual is about 200 IU/m^2 per week.

73. The method of claim 1 wherein the amount of arginine deiminase bonded to polyethylene glycol administered to the individual is about 200 IU/m^2 per week.

Incidence and Distribution of Argininosuccinate Synthetase Deficiency in Human Cancers

A Method for Identifying Cancers Sensitive to Arginine Deprivation

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BACKGROUND. Argininosuccinate synthetase (ASS) was the first of two enzymes to convert citrulline to arginine. This pathway allowed cells to synthesize arginine from citrulline, making this amino acid nonessential for the growth of most mammalian cells. Previous studies demonstrated that several human tumor cell lines were auxotrophic for arginine due to an inability to express ASS. Selective elimination of arginine from the circulation of animals with these tumors is a potentially effective anticancer treatment. The purpose of these experiments was to determine the frequency of ASS deficiency and arginine auxotrophy in a variety of human malignant tumors.

METHODS. The authors analyzed the expression of ASS by immunohistochemistry with a monoclonal antibody in a variety of human tumor biopsies. They found that the incidence of ASS deficiency varied greatly with the tumor type and tissue of origin.

RESULTS. Melanoma, hepatocellular carcinoma, and prostate carcinoma were most frequently deficient in ASS. Some human cancers were almost always positive for ASS (e.g., lung and colon carcinomas). However, other human cancers, including sarcomas, invasive breast carcinoma, and renal cell carcinoma, also were sometimes ASS deficient.

CONCLUSIONS. These data indicated that immunohistochemical detection of ASS may prove an effective means for determining ASS deficiency in malignant human tumors and for identifying patients most likely to respond to arginine deprivation therapy. Based on these results, human clinical trials using arginine- degrading enzyme therapy to treat patients with advanced melanoma or hepatocellular carcinoma have been initiated. *Cancer* 2004;100:826–33.

KEYWORDS: arginine, argininosuccinate synthetase (ASS), arginine deiminase, histochemistry, cancer, pharmacogenetics.

Amino acid deprivation therapy is an effective method to treat some cancers.[1] The best known of these treatments utilizes asparaginase to lower circulating levels of asparagine. This treatment is particularly effective for acute lymphoblastic leukemia because these cells lack asparagine synthetase and thus require asparagine.[2,3] In contrast, most normal human cells express asparagine synthetase and elimination of this amino acid is well tolerated. Therefore, there is precedence for the use of an amino acid-degrading enzyme as an effective treatment for specific forms of cancer that are auxotrophic for nonessential amino acids.

Arginine is another nonessential amino acid for humans and mice.[4,5] It can be synthesized from citrulline in two steps via the urea

cycle enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). ASS catalyzes the conversion of citrulline and aspartic acid to argininosuccinate, which is then converted to arginine and fumaric acid by ASL.

Unlike normal cells and tissues, it has long been known that various tumor cells are auxotrophic for arginine. This observation was made initially using normal cells derived from the liver, testes, and kidney. Normal cells derived from these organs grew in media depleted of arginine and supplemented with citrulline, whereas malignant cells derived from those organs did not.[6] Additional confirmation of this observation was provided by several laboratories studying tumor cell growth in vitro. Kenny and Pollock[7] and Kraemer et al.[8] observed independently that *Mycoplasma* infection frequently killed human tumor cell lines, including cervical carcinoma cells and a leukemia cell line but not normal embryonic cells. A later study by Kraemer[9] determined that the *Mcyoplasma* killed these tumor cells by depleting the tissue culture media of arginine. Schimke et al.[10] found that *Mcyoplasma* had an arginine-degrading pathway that was not present in mammalian cells–arginine deiminase (ADI), which catalyzes the conversion of arginine to citrulline. The enzymatic action of ADI was responsible for the observed in vitro killing of several diverse tumor cell lines (Table 1).

A high percentage of specific histologic types of solid human malignancies require arginine for growth and cannot utilize citrulline as a precursor for this amino acid. Sugimura et al.[11] determined that all five human melanoma cell lines tested in their laboratory were auxotrophic for arginine. We confirmed these observations and found that all 16 human melanoma and 16 human hepatocellular carcinoma (HCC) cell lines tested in vitro were auxotrophic for arginine.[12] Further analysis of these tumors demonstrated that arginine auxotrophy is a result of an inability of these tumors to express ASS.[12,13]

Much earlier work involving deficient ASS expression in human cancer focused on the use of tumor-derived cell lines. Although human melanomas and HCC have a high incidence of ASS deficiency, other human tumor cell lines may also have this genetic defect. The goals of the current study were to determine whether other human malignancies were ASS deficient and to delineate the incidence and tissue distribution of this deficiency in human tumors.

MATERIALS AND METHODS

Materials and Cell Lines

All human tissue specimens were obtained from either the National Cancer Institute (NCI) Human Tissue Cooperative Network at the University of Alabama (Birmingham, AL) or The University of Texas M. D. Anderson Cancer Center (Houston, TX). All laboratory reagents were obtained from Sigma Chemical Company (St. Louis, MO) except where indicated.

Production of Recombinant Argininosuccinate Synthetase

Human recombinant ASS was produced as previously reported[14] with minor modifications. *Escherichia coli* was grown to a density of 8 Optical Density (OD) in a fermentor before the addition of Isothiopyranoglactoside (IPTG) (1 mM), which induces the expression of the argininosuccinate synthetase gene, to the culture. The cells were grown for an additional 2 hours and harvested by centrifugation.

The cells were lysed in a buffer comprised of 300 mM of NaCl and 50 mM of sodium phosphate [pH 8.0] using a microfluidizer and were then centrifuged (20,000 × g for 20 minutes) to remove cell debris. The supernatant fluid was passed over a Ni-affinity column (Qiagen, Valencia, CA). The column was washed extensively with lysis buffer containing 0.1 M imidazole and 10% (volume/volume [v/v]) glycerol. The column was eluted with a buffer comprised of 0.3 M imidazole in 50 mM of sodium phosphate [pH 8.0]. The resulting recombinant ASS was > 99% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The recombinant ASS was cross-linked by the addition of formaldehyde (3% v/v) and used for the production of the monoclonal antibody (MoAb).

Monoclonal Antibody Production

The MoAb was made using standard laboratory techniques. The resulting clones of cells were screened initially using microtiter plates and subsequently tested for the ability to recognize ASS by Western blots and formaldehyde-fixed cells. Once a suitable clone of hybridoma cells had been identified, the cells were grown in tissue culture culture spinner flasks and the antibody (an immunoglobulin G [IgG] 2b) was purified by affinity chromatography using a protein A column (Sigma Chemical Company).

Immunohistochemistry

Four groups of melanocytic lesions were selected for immunohistochemical analysis, including benign nevi (BN) (17 biopsy specimens), dysplastic nevi (18 biopsy specimens), superficial spreading/nodular/acral/lentigo malignant melanoma (23 biopsy specimens), and subcutaneous/lymph node/visceral metastatic malignant melanoma (MMM; 31 cases). Areas for the tissue arrays were selected by reviewing the hematoxylin and eosin (H & E) slides. The 3 recipient blocks were con-

TABLE 1
Inhibition of Tumor Cell Growth by Arginine Deiminase in Tissue Culture

References	Cancer cell type
Kenny and Pollock, 1963[7]	Lymphoblastic leukemia L5178Y, murine
Kraemer et al., 1963[8] and Kraemer, 1964[9]	Lymphoblastic leukemia L5178Y, murine
Gill and Pan, 1970[30]	Lymphoblastic leukemia L5178Y, murine
van Diggelen et al., 1977[36]	A-9 cells, murine
Jones, 1981[33]	Lymphoblastic leukemia L5178Y and L1210, murine
Sasaki et al., 1984[35]	Breast carcinoma FM3A cells (derived from C3H Mouse mammary carcinoma)
Miyazaki et al., 1990[20]	Hepatoma HLE, human tongue
	Squamous carcinoma HSC-4, human
	Cervix squamous carcinoma CaSki, human
	Lung adenocarcinoma A549, human nasal
	Adenocarcinoma KB, human
	Glioblastoma T98G, human
Sugimura et al., 1990[11]	Melanoma Mewo, VMRC-MELG, A375, G361, C32TG, human melanoma B16F1, mouse
	T-lymphoma Jurkat, human
	T-cell leukemia TL-Mor and MT-2, human
	B-cell lymphoma Raji and Manaca, human
	Histiocytic lymphoma U937, human
	Neuroblastoma SK-N-MC, human
Gonzalez and Byus, 1991[17]	Skin carcinoma induced by 12-O-tetradecanoyl-phorbol-3-acetate
Sugimura et al., 1992[13]	Melanoma A375, G-361, Mewo, VMRC-MELG and C32TG, human
Takaku et al., 1992[21]	Melanoma B16, murine
	Hepatoma MH134, murine
	Colon carcinoma Colon 26, murine
	Fibrosarcoma Meth A, murine
	Sarcoma S-180, murine
	Leukemia L1210, murine
Takaku et al., 1993[22]	Hepatoma MH134, murine
	Colon carcinoma Colon 26, murine
	Sarcoma S-180, murine
	Leukemia L1210, murine
Misawa et al., 1994[34]	Hepatoma MH134, murine
	Fibrosarcoma Meth A, murine
Takaku et al., 1995[25]	Hepatoma MH134, murine
	Fibrosarcoma Meth A, murine
Gong et al., 1999[31]	Osteosarcoma SaOS, human
	Neuroblastoma (SH-EP and WAC2), human
	Retinoblastoma (Y-79), human
Gong et al., 2000[32]	Lymphoblastic leukemia, human
	Myeloid leukemia, human
Kenny 1963	Lymphoblastic leukemia L5178Y, murine
Kraemer 1963 and 1964	Lymphoblastic leukemia L5178Y, murine
Gill 1970[30]	Lymphoblastic leukemia L5178Y, murine
van Diggelen 1977[36]	A-9 cells, murine
Jones 1981[33]	Lymphoblastic leukemia L5178Y and L1210, murine
Sasaki 1984[35]	Breast carcinoma FM3A cells (derived from C3H mouse mammary carcinoma)
Miyazaki 1990	Hepatoma HLE, human
	Tongue squamous carcinoma HSC-4, human
	Cervix squamous carcinoma CaSki, human
	Lung adenocarcinoma A549, human
	Nose adenocarcinoma KB, human
	Glioblastoma T98G, human
Sugimura 1990	Melanoma Mewo, VMRC-MELG, A375, G361, C32TG, human
	Melanoma B16F1, mouse
	T-lymphoma Jurkat, human
	T-cell leukemia TL-Mor and MT-2, human
	B-cell lymphoma Raji and Manaca, human
	Histiocytic lymphoma U937, human
	Neuroblastoma SK-N-MC, human
Gonzalez 1991	Skin carcinoma induced by 12-O-tetradecanoyl-phorbol-3-acetate
Sugimura 1992	Melanoma A375, G-361, Mewo, VMRC-MELG and C32TG, human
Takaku 1992	Melanoma B16, murine
	Hepatoma MH134, murine
	Colon carcinoma Colon 26, murine
	Fibrosarcoma Meth A, murine
	Sarcoma S-180, murine
	Leukemia L1210, murine
Takaku 1993	Hepatoma MH134, murine
	Colon carcinoma Colon 26, murine
	Sarcoma S-180, murine
	Leukemia L1210, murine
Misawa 1994[34]	Hepatoma MH134, murine
	Fibrosarcoma Meth A, murine
Takaku 1995	Hepatoma MH134, murine
	Fibrosarcoma Meth A, murine
Gong 1999[31]	Osteosarcoma SaOS, human
	Neuroblastoma (SH-EP and WAC2), human
	Retinoblastoma (Y-79), human
Gong 2000[32]	Lymphoblastic leukemia, human
	Myeloid leukemia, human

structed with 0.6-mm cylinders (small biopsies) and 1.0-mm cylinders (larger specimens), including 1 or 2 cylinders from each case. This procedure allowed preservation of most of the lesion in the original paraffin blocks. Two control biopsy specimens (one BN case and one MMM case) were included in all three blocks. One H & E slide and 5-μm unstained slides were cut from the block. All other sections were obtained from the National Cancer Institute Human Tissue Cooperative Network at the University of Alabama. Slides containing sections from human tumors were baked at 65 °C for 24 hours to attach the sections to the slides, then cooled to room temperature. Next, the paraffin was removed with xylene and the sections were rehydrated by immersion in ethanol and then in water.

Antigen retrieval techniques were performed by microwave heating at a high setting for 15 minutes the citrate buffer or by immersing the slides into a solution of 1 mM ethylenediaminetetraacetic acid at 95 °C for 15 minutes. There was no discernible difference in either of these two methods (data not shown). The slides were rinsed in phosphate-buffered saline (PBS) (130 mM of NaCl and 50 mM of sodium phosphate [pH 7.5]). Nonspecific antibody binding sites were blocked by incubation of the slides in PBS containing 5% fetal bovine serum (v/v) and 0.05% Tween 20 (v/v). The slides were incubated with the MoAb at a concentration of 0.05 μg/mL and reacted with a kit from Vector Laboratories (Burlingame, CA) as suggested by the manufacturer. All slides were counterstained using hematoxylin.

Protein Determinations

The protein concentration of all solutions was determined with Bradford reagent (BioRad, Richmond, CA) as suggested by the manufacturer. Bovine serum albumin was used as the standard (Sigma Chemical Company).

SDS-PAGE

The SDS gels were 10–20% gradient gels obtained from Novex (Rockford, IL). They were stained with Coomassie blue (Gel Code Blue; Pierce, Rockford, IL).

Western Blot Analysis

To demonstrate the specificity of the MoAB, Western blot analysis was performed using standard laboratory techniques, implementing a secondary antibody (biotin-conjugated goat anti-mouse IgG; Sigma Chemical Company) and Vectastain ABC reagent as suggested by the manufacturer (Vector Laboratories).

RESULTS

Previous results from Sugimura et al.[11] and Ensor et al.[12] indicated that human melanomas and HCC were unable to survive in arginine-deficient media because they were unable to synthesize ASS messenger RNA (mRNA). We confirmed this using reverse transcription-polymerase chain reaction (RT-PCR). Although this method for detection of ASS was suitable for use in tissue culture cells, evaluation of human tumor biopsy specimens is confounded by the presence of normal tissue cells. Several human melanoma biopsy specimens were also tested for ASS mRNA by RT-PCR. However, unlike data obtained from the melanoma cell lines, all tumor biopsy specimens contained mRNA encoding ASS (data not shown). The source of the mRNA encoding ASS was most likely mRNA contamination from infiltrating microvessels and normal connective tissue in the biopsy samples.

To determine whether the ASS detected by RT-PCR originated from either the melanoma or contaminating cells in the biopsy specimens, an antibody to ASS was made. The antigen used for the immunization of the mice was purified to homogeneity as determined by SDS-PAGE. The MoAb recognizes only a single protein of the correct molecular weight when tested by Western blot analysis (Fig. 1).

Using the purified MoAb to ASS, immunohistochemistry was performed to evaluate the expression of ASS in a large series of 89 benign and malignant human melanocytic lesions. There was no expression of ASS in either BN or dysplastic nevi, primary melanoma, or metastatic melanoma specimens. Therefore, the histochemical data obtained with the melanomas were consistent with the RT-PCR data obtained from the human melanoma tumor cell lines.[12]

To determine the incidence of ASS deficiency in a variety of human tumor specimens, a large number of biopsy specimens were obtained and labeled using this MoAb. In each section, the endothelium lining the vasculature served as a positive control. The result from this survey of biopsies is shown in Table 2. The incidence of ASS deficiency was highly dependent on the type of tumor specimen being analyzed. The lung and colon carcinoma biopsy specimens showed ASS expression (Fig. 2C,D). Other malignant tumor biopsy specimens, such as tumor specimens of the breast, expressed ASS in some but were completely deficient in other specimens. Most of the breast biopsy specimens expressed ASS (Fig. 2A). However, approximately 10% of the breast carcinoma biopsy specimens were ASS deficient (Fig. 2B). Prostate carcinoma and melanoma biopsy specimens were ASS deficient (Fig. 2E,F).

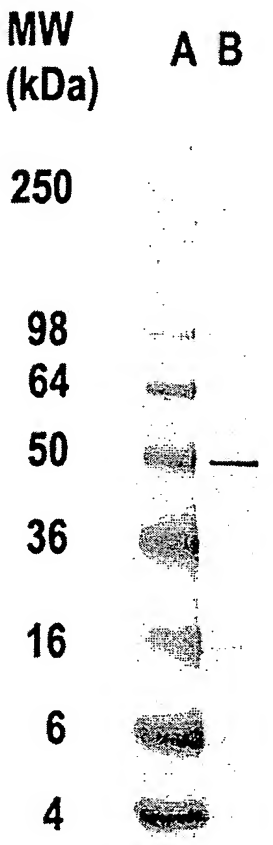

FIGURE 1. Western blot analysis of tumor cell lysate. The specificity of the monoclonal antibody to argininosuccinate synthetase (ASS) was tested. Cell-free extract was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred to a membrane and probed with affinity-purified anti-human ASS antibody. (Lane A) Protein molecular weight markers. (Lane B) Cell extract.

DISCUSSION

It has long been known that arginine is required for the growth of some tumors. For example, Gilroy in 1930[15] and Yeatman et al. in 1991[16] demonstrated that mice fed an arginine-enriched diet developed tumors that grew faster and larger than mice fed a normal diet. Conversely, mice fed an arginine-deficient diet have reduced tumor growth.[16,17] Therefore, there is a long history of evidence in the nutritional literature indicating a requirement for arginine in the growth of some tumors.

Independent of these nutritional reports, a considerable number of studies regarding the deleterious effects of *Mycoplasma* contamination on the viability of tumor cells in tissue culture have been reported. It was primarily the work of Kraemer in 1964[9] that demonstrated that the inhibitory effects of *Mycoplasma* contamination on these tumors could be overcome by the addition of excess arginine to the cell cultures. The Kraemer study was extended by Simberkoff et al. in 1969,[18] who determined that it was the ADI in the *Mycoplasma* that was responsible for elimination of arginine from the culture medium. This finding did not receive the recognition it may have deserved as it was widely known that most mammalian cells do not require arginine for growth in tissue culture because media that contain citrulline in place of arginine can most often support normal growth.[6,10] In 1979, Sun et al.[19] demonstrated definitively that some tumor cells are unable to utilize citrulline for growth. However, most of these investigators concluded that *Mycoplasma* contamination of tissue culture should be avoided. Further study in this area ended without addressing the arginine-dependent growth of the cancer cells.

Sugimura et al.,[11,13] Miyazaki et al.,[20] and Takaku et al.[21,22] tested ADI in models of melanoma, HCC, and other tumor cell lines and found that the enzyme produced tumor cell death. However, it was primarily Sugimura et al. who first showed that a number of melanoma cell lines were sensitive to arginine deprivation therapy with an enzyme like ADI. This finding suggested that this disease would be a target for arginine depletion. Our goal was to determine the relative incidence of arginine auxotrophy in a large series of of human melanomas and HCC. We focused primarily on these tumors, in part because of the work by these investigators, but also because of the observation that levels of free arginine are increased 5–20-fold in ani-

TABLE 2
Incidence and Distribution of ASS Deficiency in Human Tumor Biopsy Specimens

Tumors	No.	-ASS	Foci only +
Breast (invasive ductal carcinoma)	56	5	0
Colon (adenocarcinoma)	46	1	0
Kidney (renal cell carcinoma)	21	4	2
Lung (squamous)	26	1	2
Lung (adenocarcinoma)	20	0	0
Hepatocellular carcinoma	51	51	0
Melanoma	119	119	0
Ovarian	45	2	0
Prostate	13	13	0
Sarcoma	27	4	2
Leiomyosarcoma	9	1	1
Liposarcoma	8	3	0
Synovial sarcoma	10	0	1
Seminoma	12	2	0
Stomach (adenocarcinoma)	6	0	0

ASS: argininosuccinate synthetase; -: negative; +: positive.

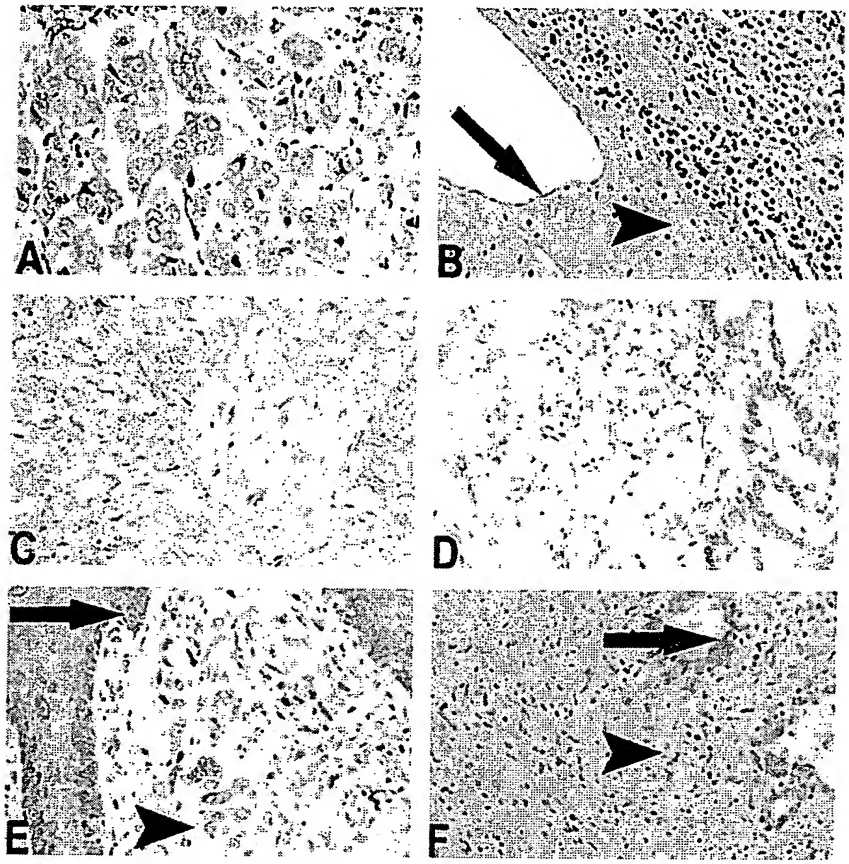

FIGURE 2. Immunohistochemical analysis of arginosuccinate synthetase (ASS) expression in various tumors. Human tumor sections were treated. ASS expression is dependent on the tumor type. (A and B) Breast tumors. (C) Lung tumors. (D) Colon tumors. (E) Melanoma tumors. (F) Prostate tumors. ASS is expressed in epithelial and endothelial cells (arrow) whereas tumor cells do not express ASS (arrowhead).

mals bearing HCC.[23] Furthermore, the urea cycle is depressed in most HCC cell lines. Therefore, these cells must obtain arginine from the circulation. Depletion of plasma arginine would be expected to result in growth inhibition of these tumors in vivo. We were surprised that all human melanoma and HCC cell lines tested were quite sensitive to ADI inhibition in vitro.[12] These results were in large part a continuation of the work performed by others (Table 1) who demonstrated that *Mycoplasma* infection of, or addition of ADI to, cancer cell lines can inhibit their growth, with perhaps the most striking inhibition being observed with human HCC (HLE) and melanoma (VMRC) cell lines.[20]

Previous investigators have demonstrated in tissue culture that arginine deprivation by ADI results in tumor cell death (Table 1). Others have also depleted arginine from cell culture media and demonstrated inhibition of cervical carcinoma (HeLa), squamous cell carcinoma (A431), breast carcinoma (MCF7, ZR75-1), ovarian carcinoma (PEO), prostate carcinoma (PC3), colon carcinoma (WiDr), lung carcinoma (A549), osteosarcoma (KHOS/NP, U2OS, Saos 2), glioma/astrocytoma (GO-C-CCM), glioblastoma (U-870-MG), premyelocytic leukemia (HL 60), and lymphoblastic leukemia (MOLT4).[24] In addition, they also demonstrated inhibition of murine melanoma (B16-F10) growth. However, not all cancers have responded to arginine deprivation. For example, human tongue squamous cell carcinoma (HSC-3) and cervix squamous cell carcinoma (C41) responded minimally to reduced arginine levels in media.[20] Similarly, ape T-cell lymphoma (MLA144), mouse macrophage (P388D1), and mouse mastocytoma (P815) cells were not sensitive.[11]

Citrulline is converted to arginine by the sequential action of two intracellular enzymes, ASS and ASL. Sugimura et al.[13] evaluated RNA samples from several melanoma cells and found that ASS was not expressed. They hypothesized that the absence of ASS was responsible for the arginine auxotrophy of these cells. We extended the research of Sugimura et al. by evaluating not only the expression of ASS but also the expression of ASL. Consistent with the observations of Sugimura et al., we were unable to detect ASS mRNA but were able to detect ASL mRNA.[12,13] To demonstrate conclusively that ASS deficiency caused sensitivity to ADI treatment, cells were transfected with an

expression plasmid containing ASS complementary DNA. The transfected cells were much more resistant to ADI than the parental cells. Therefore, the ADI sensitivity of the cells is a result of this ASS deficiency. To our knowledge, it is not currently known why these cells are unable to express ASS. Initial evaluation of the gene indicated no obvious defect in its structure or the promotor region. We are currently pursuing this area of research. Currently, the cause of this defect remains unknown.

Regardless of the underlying mechanism that renders some tumors unable to synthesize arginine from citrulline, it is possible to utilize arginine-degrading enzymes as an effective means of eliminating the growth of these tumors in mice.[12,21,25] Recently, we initiated human clinical testing of one such arginine-degrading enzyme, termed ADI-SS $PEG_{20,000mw}$,[26] in patients with melanoma and HCC. A once-a-week injection of this enzyme in mice and humans is sufficient to eliminate all detectable arginine from the circulation (data not shown) and to block nitric oxide production.[12,27,28] This treatment is well tolerated in mice and preliminary results from human clinical testing indicate that this therapy is also well tolerated in humans. The initial clinical testing of this drug is limited to patients with melanoma and HCC because of the high incidence of ASS deficiency observed in these tumors. However, the results indicate that this therapy may have utility in the treatment of other human tumors that are ASS deficient. The immunohistochemical detection method may ultimately identify tumors that will be responsive to arginine deprivation therapy, thus providing a pharmacogenetic approach to drug development and therapy.[29]

REFERENCES

1. Cooney DA, Rosenbluth RJ. Enzymes as therapeutic agents. *Adv Pharmacol.* 1975;12:1985–1962.
2. Asselin BL. The three asparaginases. Comparative pharmacology and optimal use in childhood leukemia. *Adv Exp Med Biol.* 1999;457:621–629.
3. Müller HJ, Boos J. Use of L-asparaginase in childhood ALL. *Crit Rev Oncol/Hematol.* 1998;28:97–113.
4. Rogers QR. Species variation in arginine requirements. Proceedings from a symposium honoring Willard J. Visek—from ammonia to cancer and gene expression. Special Publication 86, Agriculture Experiment Station, University of Illinois, April 4–5, 1994:9–21.
5. Barbul A. Arginine: biochemistry, physiology, and therapeutic implications. *J Parenteral Enteral Nutr.* 1986;10:227–238.
6. Tycell E, Neuman RE. Growth response of stable and primary cell cultures to L-ornithine and L-citrulline and L-arginine. *Exp Cell Res.* 1960;20:84–91.
7. Kenny GE, Pollock ME. Mammalian cell cultures contaminated with pleuropneumonia-like organisms. I. Effect of pleuropneumonia-like organisms on growth of established cell strains. *J Infect Dis.* 1963;112:7–16.
8. Kraemer PM, Defendi V, Hayflick L, Manson LAl. *Mycoplasma* (PPLO) strains with lytic activity for murine lymphoma cells in vitro. *Proc Soc Exp Biol Med.* 1963;112:381–387.
9. Kraemer PM. Interaction of *Mcyoplasma* (PPLO) and murine lymphoma cell cultures: prevention of cell lysis by arginine. *Proc Soc Exp Biol Med.* 1964;115:206–212.
10. Schimke RT, Berlin CM, Sweeney EW, Carroll WRI. The generation of energy by the arginine dihydrolase pathway in *Mycoplasma hominis* 07. *J Biol Chem.* 1964;241:2228–2236.
11. Sugimura K, Ohno T, Fukuda S, Wada Y, Kimura T, Azuma I. Tumor growth inhibitory activity of a lymphocyte blastogenesis inhibitory factor. *Cancer Res.* 1990;50:345–349.
12. Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA. Pegylated arginine deiminase (ADI-SS PEG20,000 mw) inhibits human melanomas and hepatocellular carcinomas in vitro and in vivo. *Cancer Res.* 2002;62:5443–5450.
13. Sugimura K, Ohno T, Kusuyama T, Azuma I. High sensitivity of human melanoma cell lines to the growth inhibitory activity of *Mcyoplasma* arginine deiminase in vitro. *Melanoma Res.* 1992;2:191–196.
14. Kobayashi SN, Terazono H, Fukushige T, Horiuchi M, Saheki T. Characterization of human wild type and mutant argininosuccinate synthase proteins expressed in bacterial cells. *Enz Protein.* 1994;48:251–264.
15. Gilroy E. The influence of arginine upon the growth rate of a transplantable tumour in the mouse. *Biochem J.* 1930;24: 589–595.
16. Yeatman TJ, Risley GL, Brunson ME. Depletion of dietary arginine inhibits growth of metastatic tumor. *Arch Surg.* 1991;126:1376–1382.
17. Gonzalez GG, Byus CV. Effect of dietary arginine restriction upon ornithine and polyamine metabolism during two-stage epidermal carcinogenesis in the mouse. *Cancer Res.* 1991;51:2932–2939.
18. Simberkoff MS, Thorbecke GJ, Thomas L. Studies on PPLO infection. V. Inhibition of lymphocyte mitosis and antibody formation by *Mcyoplasma* extracts. *J Exp Med.* 1969;129: 1163–1181.
19. Sun NC, Sun CRY, Tennant RW, Hsie AW. Selective growth of some rodent epithelial cells in medium containing citrulline. *Proc Natl Acad Sci USA.* 1979;76:1819–1823.
20. Miyazaki K, Takaku H, Umeda M, et al. Potent growth inhibition of human tumor cells in culture by arginine deiminase purified from a culture medium of a *Mycoplasma*-infected cell line. *Cancer Res.* 1990;50:4522–4527.
21. Takaku H, Takase M, Abe S-I, Hayashi H, Miyazaki K. In vivo anti-tumor activity of arginine deiminase purified from *Mycoplasma arginini. Int J Cancer.* 1992;51:244–249.
22. Takaku H, Misawa S, Hayashi H, Miyazaki K. Chemical modification by polyethylene glycol of the anti-tumor enzyme arginine deiminase from *Mycoplasma arginini. Jpn J Cancer Res.* 1993;84:1195–1200.
23. Moyer GH, Pitot HC. Static and dynamic aspects of amino acid pools in rat liver and Morris hepatomas 9618A and 7800. *Cancer Res.* 1974;334:2647–2653.
24. Scott L, Lamb J, Wheatley DN. Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells. *Br J Cancer.* 2000;83:800–810.
25. Takaku H, Matsumoto M, Misawa S, Miyazaki M. Anti-tumor activity of arginine deiminase from *Mycoplasma arginini* and its growth-inhibitory mechanism. *Jpn J Cancer Res.* 1995;86:840–846.

26. Holtsberg FW, Ensor CM, Steiner MR, Bomalaski JS, Clark MA. Poly(ethylene glycol) (PEG) conjugated arginine deiminase: effects of PEG formulations on its pharmacological properties. *J Control Rel.* 2002;80:259–271.
27. Thomas JB, Holtsberg FW, Ensor CM, Bomalaski JS, Clark MA. Enzymatic degradation of plasma arginine using arginine deiminase inhibits nitric oxide production and protects mice from the lethal effects of tumour necrosis factor α and endotoxin. *Biochem J.* 2002;363:581–587.
28. Dillon BJ, Holtsberg FW, Ensor CM, Bomalaski JS, Clark MA. Biochemical characterization of the arginine degrading enzymes arginase and arginine deiminase and their effect on nitric oxide production. *Med Sci Monit.* 2002;8:248–253.
29. Shi MM, Bleavins MR, Iglesia FA. Pharmacogenetic application in drug development and clinical trials. *Drug Met Disp.* 2001;29:591–595.
30. Gill P, Pan J. Inhibition of cell division in L5178Y cells by arginine-degrading *Mcyoplasma*: the role of arginine deiminase. *Can J Microbiol.* 1970;16:415–419.
31. Gong H, Zölzer F, von Recklinghausen G, et al. Arginine deiminase inhibits cell proliferation by arresting cell cycle and inducing apoptosis. *Biochem Biophys Res Commun.* 1999;261:10–14.
32. Gong H, Zölzer F, von Recklinghausen G, Havers W, Schweigerer L. Arginine deiminase inhibits proliferation of human leukemia cells more potently than asparaginase by inducing cell cycle arrest and apoptosis. *Leukemia.* 2000;14:826–829.
33. Jones JB. The effect of arginine deiminase on murine leukemia lymphoblasts [Ph.D. dissertation]. Oklahoma City, OK: University of Oklahoma, 1981:1–169.
34. Misawa S, Aoshima M, Takaku H, Matsomoto M, Hayashi H. High-level expression of *Mycoplasma* arginine deiminase in *Escherichia coli* and its efficient renaturation as an antitumor enzyme. *J Biotechnol.* 1994;36:145–155.
35. Sasaki T, Shintani M, Kihara K. Inhibition of growth of mammalian cell cultures by extracts of arginine-utilizing *Mcyoplasma. In Vitro.* 1984;20:369–375.
36. van Diggelen OP, Shin S-i, Phillips DM. Reduction in cellular tumorigenicity after *Mcyoplasma* infection and elimination of *Mcyoplasma* from infected cultures by passage in nude mice. *Cancer Res.* 1977;37:2680–2687.